# PCT

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(54) Title: STEREOREGULAR POLYNUCLEOTIDE-BINDING POLYMERS

(57) Abstract

A polymeric composition effective to bind, with a selected binding affinity, to a single-stranded polynucleotide containing a target sequence of bases. The composition includes non-homopolymeric, substantially stereoregular polymer molecules, each composed of a sequence of base complementary recognition moieties adapted to hydrogen bond to corresponding, in-sequence bases in such target sequence, under selected binding conditions, and a substantially stereoregular, predominantly uncharged backbone



supporting the recognition moieties at positions which allow hydrogen bonding between the recognition moieties and the corresponding in-sequence bases in the target sequence. The composition is useful in a diagnostic assay system for determination of a polynucleotide containing the target sequence; in research applications involving binding to specific target sequences in solution for the purpose of selectively altering target expression, including replication, transcription, and translation; and in therapeutic uses based on inactivation of single stranded polynucleotides *in vivo*.

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## STEREOREGULAR POLYNUCLEOTIDE-BINDING POLYMERS

### 1. Field of the Invention

The present invention relates to base-specific polynucleotide-binding polymers

### 2. Background of the Invention

Compounds capable of recognizing and binding specifically to a target base sequence in a polynucleotide have many potentially valuable applications. For example, in therapeutic uses, the compounds could act to bind to and inactivate viral genomes, or other single-stranded polynucleotide species characteristic of an infecting microorganism. In diagnostic applications, the compounds could be used to "hybridize" with analyte single-stranded polynucleotides, to detect the presence of viral or bacterial-specific nucleic acid species.

The earliest attempts to devise an agent which is capable of binding specifically to a base-specific target region of a polynucleotide were based on the concept of (Belikov, Zarotova, Grinev) derivatizing a target-specific polynucleotide with an alkylating agent, (Summerton and Bartlett (1978), J. Molec. Biol, 122:145) binding the derivatixed copound to the target single-strand RNA or DNA. Alkylation of the target strand would then bind the compound irreversible to the target. There are two major difficulties with this approach. First, the negatively charged backbone of the derivatized polynucleotide seriously restricts its transport into living cells. Second, the phosphodiester linkages in the agents are susceptible to rapid cleavage by cellular nucleases -- particularly when the gene

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inactivating agents are taken into cells via
endocytosis.

More recently, recombinant DNA methods have
allowed for the design of plasmids which carry a
anti-sense gene whose RNA transcript can recognize and
5     bind to a particular target single-strand
polynucleotide. Introduction of such a plasmid into
cells results in continuing production of anti-sense
mRNA which appears to pair with its complementary target
mRNA and thereby block its translation (Izant and
10     Weintraub (1984), Cell, 36:1007; Izant and Weintraub
(1985), Science, 229:345). A particular merit of this
approach is that successful introduction of even a
single such plasmid could potentially effect continued
inactivation of the targeted gene activity and/or
15     permanent resistance to the pathogen containing the
targeted genetic sequence. The approach is severely
limited, however, by the problems of transforming a
large number of tissue or organ cells with the plasmid
construct.
20     Attempts to design a polynucleotide binding
agent which is both readily taken up by cells and stable
against intracellular degradation have focused on
polynucleotide analogs which have uncharged or
substantially uncharged backbones. One of the earliest
25     reports of such uncharged comppounds involved a number
of polynucleotide analogs in which the normal
sugar-phosphate backbone of nucleic acids was replaced
by a polyvinyl backbone. These nucleic acid analogs
were shown to have normal Watson/Crick pairing
30     specificities with complementary polynucleotides -- but
with substantially reduced Tm values (Pitha and Pitha
(1970), Biochem. Biophys. Acta, 204:39).

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Subsequently, uncharged and substantially uncharged heteropolymeric polynucleotide analogs more nearly isostructural with nucleic acids have been prepared for the purpose of _in vivo_ inactivation of targeted single-stranded genetic sequences.  These

5  substances include both aryl nitrogen mustard derivatized (Karpova et al (1980), FEBS Letters, _122_:21) and underivatized (Richard Tullis, Personal Communication, Molecular Biosystems, Inc., San Diego) oligonucleotides having their phosphates in the

10  uncharged triester state.  A novel class of polynucleotide analogs which has recently been reported contain predominantly uncharged, atactic methylephosphonate backbone linkages, or homochiral methylphosphonate linkages alternating with charged,

15  achiral phosphodiester linkages (Miller, Yano, Yano, Caroll, Jayaraman, and Ts'o (1979), Biochem., _18_:5134; Miller, Dreon, Pulford, and Parland (1980), J. Biol. Chem., _255_:9659; Murakami, Blake, and Miller (1985), Biochem., 24:4041; Blake, Murakami, Spitz, Glave, Reddy,

20  Ts'o, and Miller (1985), _24_:6132; Miller, Agris, Aurelian, Blake, Murakami, Reddy, Spitz, and Ts'o (1985), Biochimie, _67_:769).

The above-described uncharged or substantially uncharged oligonucleotide analogs have been reported to

25  enter living cells and pair with complementary genetic sequences therein.  In particular, it has been found that optimal uptake by the cells is achieved if the compounds are designed with one or a few ionic groups per molecule, to enhance solubility in aqueous medium.

30  If the charge density on the backbone is increased significantly, however, problems of poor transport across the cell membrane are encountered.  Also, greater

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stability to nucleases is achieved if phosphodiester
linkages are avoided.

The above uncharged and substantially uncharged
polynucleotide analogs all have chiral backbone
linkages, i.e., linkages in which two possible
5  stereoisomeric forms are allowed.  As a consequence, the
backbone of the polymer produced by linking subunits has
a random sequence of left- and right- handed forms.
This type of backbone structure, which is also referred
to as an atactic backbone, is characterized by a broad
10  range of analog/target binding constants.  The mean
binding constant can be significantly reduced with
respect to the binding constant between normal
complementary polynucleotides.

For the phosphotriester and methylphosphonate-
15  linked nucleic acid analogs this binding constant
reduction and broadening presumably  derives from one
linkage isomer favoring Watson/Crick pairing of proximal
bases and the other linkage isomer inhibiting
Watson/Crick pairing of proximal bases.  This linkage
20  chirality effect on base-pairing has been well
established by studies involving two separated isomeric
forms of a methylphosphonate-linked dideoxyribonucleo-
side.  Each of these dimers was mixed with its
complementary polyribonucleotide.  One isomer was found
25  to bind with a Tm of 19.8°C while the other isomer had a
corresponding Tm value of only 15.4°C (Miller, Dreon,
Pulford, and Parland (1980), J. Biol, Chem, 255:9659).
These studies were further extended to examine Tm
binding values in homoisomeric decamers formed by
30  linking the isomeric methylphosphonate dimers with
normal phosphodiester linkages.  The decamer formed from
dimers having the preferred isomeric methylphosphonate
linkages paired with its complementary genetic sequence

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with a Tm of 33°C.  By contrast, the corresponding decamer containing the other isomeric form of methylphosphonate linkages failed to pair with its complementary genetic sequence even at 0°C (Miller, Dreon, Pulford, and Parland (1980), J. Biol. Chem. 255:9659).

On theoretical grounds there is reason to believe that for a sequence-specific polynucleotide-binding agent to be of therapeutic and/or diagnostic value, it should pair with its complementary target sequence with a substantially uniform binding constant. The rationale here is that if a given preparation contains multiple molecular species, each with its own separate target binding constant, then those species having lower binding constants will contribute little to the target binding activity while some of those having significantly higher binding constants also form moderately stable mispaired complexes with nontarget sequences.  In a therapeutic context any significant binding to nontarget sequences has the potential of generating toxic side effects, while in a diagnostic context such binding is expected to lead to high backgrounds and/or false positives.

In principal, using currently available technologies it should be possible to prepare the foregoing charged or partially charged and chirally linked nucleic acid analogs having substantially uniform target binding constants.  One method for accomplishing this would involve binding the the polymer solution, which contains polymer species with a wider range of Tm values for a given target sequence, to an affinity column having the target sequence in an immobilized form.  The analog is then eluted with with a gradient of an eluant, such as formamide, which will release

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increasingly more tightly bound analog at higher eluant
concentrations, and/or by eluting while slowly
increasing the temperature.  A narrow cut of the eluant
should contain primarily those molecules having a
substantially uniform target binding constant.
5 Achieving greater binding homogeneity with this method
obviously means reducing the final yield of usable
polymer substqntially.  Alternatively, it is possible
that uncharged oligonucleotide analogs having tactic
linkages (i.e., all molecules having the same sequence
10 of chiral centers) could be prepared by means of a
geometric assembly procedure in which one of the two
stereoisomers formed at each subunit addition step is
retained, and the other discarded.  The limitation of
this procedure, of course, is that at least half of the
15 material is discarded at each subunit addition step, so
that the yield can be at most $1/2^n$ of the starting
material, where n is the number of rounds of couplings
required in the synthesis.

Thus, although it may be possible to prepare
20 uncharged polynucleotide analogs having chiral
intersubunit linkages which pair with their
complementary target sequences with a substantially
uniform binding constant, nevertheless, the synthesis
and purification of such chirally linked polymers would
25 be time-consuming and yields would necessarily be poor.


3.  Summary of the Invention
It is therefore one general object of the
invention to provide a base-specific
30 polynucleotide-binding composition which substantially
overcomes problems and limitations associated with prior
art polynucleotide-binding agents.

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Related objects of the invention include:

(a) Providing such a composition whose polymer molecule components all have substantially the same binding affinity with respect to a target sequence in a single-stranded polynucleotide;

(b) Providing such a composition which can be designed for targeting against virtually any nucleotide target sequence within a length range of between about 5-30 base pairs, and which can be prepared in relatively high yield;

(c) Providing such a binding composition having a desired binding affinity for a selected target sequence; and

(d) Providing such a binding compound which is suitable for a variety of diagnostic and therapeutic applications which take advatage of the of the stereoregular, uncharged or substantially uncharged nature of the backbone linkages in the compound.

The invention includes a polymeric composition designed to bind, with a selected binding affinity, to a single-stranded polynucleotide containing a target sequence of bases. The composition includes non-homopolymeric, substantially stereoregular polymer molecules of the form:

$$R_1 \quad\quad R_2 \quad\quad R_3 \quad\quad\quad R_n$$

$$B \sim B \sim B \sim \ldots B,$$
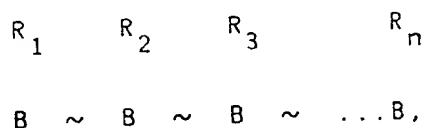
where:

(a) $R_1$-$R_n$ are recognition moieties selected from purine, purine-like, pyrimidine, and pyrimidine like heterocycles effective to bind by Watson/Crick

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pairing to corresponding, in-sequence bases in the target sequence;

(b) n is such that the total number of Watson/Crick hydrogen bonds formed between a polymer molecule and target sequence is at least about 15;

(c) B ~ B are backbone moieties joined by chemically stable, substantially uncharged, predominantly achiral linkages;

(d) the unit length of backbone moieties is 5-7 atoms if the backbone moieties have a cyclic structure, and 4-6 atoms if the backbone moieties have an acyclic structure; and

(e) the backbone moieties support the recognition moieties at positions which allow Watson/Crick base pairing between the recognition moieties and the corresponding, in-sequence bases of the target sequences.

In a preferred embodiment for use in therapeutic applications, the polymeric molecules, have a small number of charges, to increase compound solubility in aqueous medium. The charges may be attached at end regions of the molecules or at achiral charged backbone linkages.

The polymer molecules are constructed, according to the method of synthesis of the invention, by first selecting a sequence of bases in the target polynucleotide. The sequence is typically between about 5-32 bases, and preferably between about 8-20 bases, corresponding to the number of subunits in the final binding polymer. The binding affinity of the binding polymer for the target polynucleotide can be increased by increasing the number of target-sequence bases (and correspondingly, the number of subunits in the binding polymer). Polymer subunits of the form $B-R_i$ and/or

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B-R$_j$ are then coupled in a defined sequence corresponding to the sequence of bases in the target-specific sequence. Here R$_i$ and R$_j$ are recognition moities, as defined above, which are capable of forming Watson/Crick base pairing with corresponding
5  polynucleotide bases through either two or three hydrogen bonds, respectively. The binding affinity or the Tm of the binding polymer for the target polynucleotide can be selectively varied according to the ratio of R$_i$ to R$_j$ recognition moieties used in
10  forming the polymer, with a greater percentage of R$_i$ recognition moieties producing a lower binding affinity for the target polynucleotide.

The polymers are useful in a diagnostic system for detecting an analyte polynucleotide having a defined
15  target base sequence. The system includes a support reagent composed of the polymer molecules of the invention linked to a solid support. In one embodiment of the invention, the polymer binding molecules are predominantly uncharged. In another embodiment, the
20  backbone moieties are joined by achiral charged linkages, such as phosphodiester linkages, alternating with one or more uncharged achiral linkages.

In practicing the assay method, a polynucleotide analyte is added to the diagnostic
25  reagent under conditions in which the analyte is in a single-strand form and which allow sequence-specific pairing between the analyte and the reagent polymers. After the analyte/polymer annealing reaction, the reagent is washed to remove unbound test material. The
30  reagent and bound analyte are then reacted with a oligocationic reporter which is designed to bind by charge attraction to the charged, phosphodiester linkages of the bound analyte, but not to the uncharged

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or substantially uncharged polymer molecules.  One
feature of the system is that the ratio of bound
reporter to bound analyte polynucleotides (the number of
reporter molecules bound per analyte molecule) can be
$10^2-10^4$, giving a highly sensitive assay.

5          In therapeutic applications, the polymer
composition is designed to inhibit expression of a
selected target single-stranded polynucleotide, such as
an RNA virus.  Here the polymer is preferably
constructed to bind spsecifically to the target

10    polynucleotide, with the minimum binding needed to
produce the desired inhibition of target expression, to
minimize non-specific binding effects.
           These and other objects and features of the
present invention will become more fully apparent when

15    the following detailed description of the invention is
read in conjunction with the accompanying figures.


Brief Description of the Figures
           Figure 1 shows preferred purine and pyrimidine

20    structures used in forming polymer molecules of the
invention;
           Figure 2 shows preferred purine-like and
pyrimidine-like recognition moieties used in forming the
polymer molecules;

25          Figure 3 shows preferred cyclic backbone
moities used in forming the polymer molecules;
           Figure 4 shows preferred acyclic backbone
moieties used in forming the polymer molecules;
           Figures 5A and 5B illustrate two preferred

30    subunit assembly schemes for coupling $N_1---N_2$ type
subunit backbones;
           Figure 6 shows the backbone structures of
subunit dimers A-A through D-D formed in accordance with

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the methods illustrated in Figures 5A and 5B, and two
cyclic backbone structures formed in accordance with the
methods illustrated in Figures 7A and 7B;

Figures 7A-7C illustrate three preferred
subunit assembly schemes for coupling $N_1$---E type
subunit backbones; and

Figure 8 shows preferred acyclic backbone
structures of subunit dimers formed in accordance with
the methods illustrated in Figures 7A-7C;

### Detailed Description of the Invention

The polymeric composition of the invention is
designed to bind, with a selected binding affinity, to a
polynucleotide containing a target sequence of bases.
The composition is composed of non-homopolymeric,
substantially stereoregular molecules or species of the
form:

$$R_1 \quad R_2 \quad R_3 \quad\quad R_n$$

$$B \sim B \sim B \sim \ldots B,$$
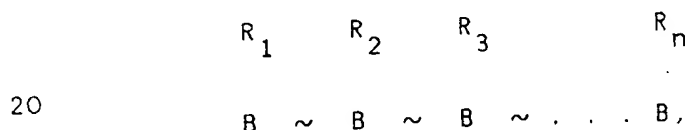
where $B-R_i$ are a series of base-specific subunits
containing a backbone moiety B and a recognition moiety
$R_i$ which is selected to bind specifically by
Watson/Crick base pairing to a corresponding,
in-sequence base in the target sequence, and the
subunits are joined through their backbone moieties
predominantly achiral, substantially uncharged bonds.
The design and selection of suitable subunits for use in
constructing the polymer species will be be described in
Section I below.  Methods for coupling subunits through
achiral linkages are described in Section II, with

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subunit-protective group strategies involved in subunit
synthesis being discussed in Section II.

  The polymer species are synthesized by
sequential subunit coupling, to form polymer molecules
of a selected length and sequence.  As will be described
5 in Section IV, the length of the target sequence and
polymer sequence is selected to achieve a desired
binding specificity.  The binding affinity of the
polymer for the target can be selectively varied by
several strategies discussed in Section IV, including
10 the choice of recognition moieties which are capable of
forming either three (for greater binding affinity) or
two (for lesser affinity) base-paired hydrogen bonds
with the corresponding target base.  The resulting
composition contains polymer species or molecules, all
15 having substantially the same sequence of subunits and
substantially the same sequence of intersubunit linkage
types.  In functional terms, all of the polymer species
have substantially the same binding affinity for the
target polynucleotide.  Methods for assembling the
20 polymer, once a desired subunit sequence is selected,
are given in Section IV.

  The polymer composition is useful in a novel
solid-phase diagnostic system which is described in
Section IV.  This system is based on the binding of
25 analyte polynucleotide molecules to support-bound
polymer molecules and subsequent elctrostatic attachment
of multiple polycationic reporter molecules to the
polynucleotide, to give an amplified reporter signal for
each bound analyte molecule.  The polymer is also useful
30 in a variety of solution applications,  including
therapeutic applications, which are also considered in
Section IV.

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I.  Subunit Structure

A.  The Recognition Moiety

The design of the subunits $B-R_i$ which are
suitable for use in forming the polymer species of the
invention involves a number of structural and/or
stereochemical criteria which must be met both by the
backbone and recognition moieties and by the linkage
between the two.  The design requirement of the
recognition moiety will be considered first.

The recognition moiety of each subunit must
provide two or three hydrogen-bonding groups held in an
unambiguous configuration adapted for hydrogen bonding
to two or three of the Watson/Crick hydrogen bonding
sites on a specified in-sequence base of the target
genetic sequence.  To avoid undesired mispairing between
recognition moiety and its corresponding target base,
under the conditions of use, (1) the tautomeric state of
the recognition moiety should be relatively stable, and
(2) the recognition moiety should have a structure which
provides a relatively rigid arrangement of the hydrogen-
bonding groups.  Such rigidity is best afforded by a
ring structure having the polar hydrogen-bonding groups
either forming part of the ring or directly attached to
the ring.

The preferred recognition moiety structures
include purine, purine-like, pyrimidine, and
pyrimidine-like structures which are designed to form
Watson/Crick base pairing, through either two or three
hydrogen bonds, with selected polynucleotide bases.  The
group of subunits used in polymer synthesis includes at
least two recognition moieties which are base-specific
for different polynucleotide bases, and preferably one
or more recognition moieties for each of the four bases

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in a natural DNA or RNA polynucleotide.  Also, as will
be seen below, it is desirable to provide one group of
recognition moieties, $R_i$,  which are capable of
base-pairing with nucleotide bases through two hydrogen
bonds, and a second group, $R_j$ which are capable of
5   binding with the same bases through three hydrogen
bonds.  Figure 1 shows exemplary purine and pyrimidine
type recognition moieties.  The purine structures 1 and
2 are designed to bind to thymine or uracil bases,
structures 3-6, to guanine bases; structures 7-9, to
10  cytosine bases, and structures 10-12, to adenine bases.
Structures 1, 4, 5, 6, 8, and 10-12 are $R_i$ type
moieties adapted to bind to corresponding in-sequence
bases through two hydrogen bonds, and the remaining
structures are $R_j$ type moieties which form three
15  hydrogen bonds on base pairing.  As will be seen below,
purine and pyrimidine nucleosides are useful in
synthesizing a variety of other subunits which are
suitable for use in polymer synthesis.  These subunits,
modified if necessary with amine protective groups, can
20  be obtained from commercial sources, or prepared
according to known methods, such as those described or
referenced in Example 1.

        A number of purine-like or pyrimidine-like
structures in which the recognition moiety is joined to
25  the backbone through an annular carbon are shown in
Figure 2.  These structures have base pairing
specificities and hydrogen bonding properties like those
of the analogous structures shown in Figure.  Although
the binding properties of a polymer containing a
30  carbon-linked recognition moiety may not be
significantly different from that of a polymer whose
recognition moities are nitrogen-linked, as in Figure 1,
subunits incorporating the carbon-linked moieties

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generally require a greater synthetic effort than do
subunits containing preformed purine and pyrimidines.
However, for some subunit syntheses, such as the
synthesis of the achiral acyclic backbone subunits
described in Example 10, the carbon-linked recognition
5  moieties provide a useful starting material.

B.  The Backbone Moiety
        The backbone moiety of each subunit has the
general form $N_1$---E, or $N_1$---$N_2$, where $N_1$ and
10  $N_2$ are nucleophilic groups and E is an electrophilic
group.  Based on ease of subunit coupling and required
stability of the resultant intersubunit linkage, as
discussed below, the preferred nucleophilic groups are
amino, hydroxyl and hydrazino; and the preferred
15  electrophilic groups, and/or electrophilic linking
agents, are derivatives of carbonic, thiocarbonic,
carboxylic, and sulfonic acids.
        Backbone moieties can have either a cyclic or
an acyclic structure.  While the total number of
20  possible backbone moiety structures is very large,
nevertheless, only a rather limited number of structures
are of actual practical value due to a number of
factors.  The initial procedure by which promising
backbone moieties were selected was as follows.
25      As a first conditions, only those cyclic
backbone moieties were considered which consisted of, or
could be readily derived from, deoxyribose or ribose.
This limitation is a practical one and reflects the
difficulty and corresponding greater expense of de novo
30  synthesizing ring structures having multiple chiral
centers.  In general prospective backbone moieties and
intersubunit linkages expected to be suitable for
polymers were selected on the basis of one or more of

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the following factors:  feasibility of synthesis based
on known reactions; expected ease of synthesis from
available starting materials; simplicity of structure;
and expected stability of the final backbone.

5        Initial screening of promising backbone
moieties and intersubunit linkages was performed as
follows.  Space-filling CPK molecular models of duplex
DNA and RNA were constructed according to parameters
determined by x-ray diffraction of oligodeoxyribo
10   nucleotides in the B-form and oligonucleotides in the
A-form.  In each of these constructed duplexes one of
the two sugar-phosphate backbones was removed.  Next,
each prospective backbone was attached, if possible, to
the sites on the bases form which the original
sugar-phosphate backbone had been removed.  Each
15   resulting polynucleotide/polymer duplex was then
examined for coplanarity of the Watson/Crick base-pairs,
torsional and angle strain in the prospective polymer
backbone, degree of distortion imposed on the nucleic
acid strand, and interstrand and intrastrand nonbonded
20   interactions.  Special attention was paid to whether or
not each amide-containing backbone could readily adopt a
conformation wherein its amide moieties were planar.
This is important because of the substantial energy cost
required to force an amide into a nonplanar conformation.
25        These initial studies verified that the
required unit backbone length (i.e., the $N_1$---E
spacing in an $N_1$---E or activated $N_1$---$N_2$-E
subunit) is 5-7 atoms for backbone moieties constituting
or derived from deoxyribose or ribose (cyclic backbone
30   structures), with a 6-atom length being optimal.
Subunit structure judged acceptable in the
above modeling studies were then assessed for
feasibility of synthesis (on the basis of key synthetic

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reactions reported in the literature, or reactions
carried out on model compounds, and/or via actual
synthesis of the subunits) and stability of the
assembled polymer backbone (preliminary stability
studies were generally carried out with suitably linked
5    model compounds or subunit dimers).  These types of
studies were used to further restrict the number of
candidate backbone structures.  From this restricted
candidate pool the cyclic backbone structures A-G shown
in Figure 3 were ultimately selected as preferred
10    structures.

In this figure, subunits A-D containing
$N_1$----$N_2$ type cyclic backbone moieties include:
2'-deoxyribonucleosides (structure A);
2'-deoxyribonucleosides substituted at the 5' and 3'
15    positions with an amino group (structures B and C,
respectively); and morpholino derivatives of
ribonucleosides (structure D).  Subunits containing
$N_1$---E-type backbone moieties include
2'-deoxyribonucleosides substituted at their 5'
20    positions with one and two-carbon acids (structures E
and F, respectively); and N-aminomorpholino derivatives
of ribonucleosides substituted at their 5' positions
with carboxylic acid (structure G) or sulfonic acid
(structure H).
25           The same molecular modeling approach applied to
acyclic (unbranched chain) backbone moieties showed that
unit backbone lengths ranging from 4-6 atoms are
acceptable, in terms of polymer conformation in
base-pair hydrogen bonding to single-stranded
30    polynucleotide, with a 5-atom backbone length being
optimal.  This is in contrast to cyclic backbones, where
a 6-atom unit backbone length is optimal.  Further, the
modeling work showed that the annular recognition moiety

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cannot be directly attached to the linear backbone
moiety without severely inhibiting of coplanarity of the
complementary bases and introducing undesirable
interactions between the recognition moiety and the
backbone the minimum binding strain occurred when the
5   recognition moiety was linked to the backbone moiety by
a one-atom spacer.  Two-atom, but not three-atom,
spacers may be tolerated, although increased degrees of
freedom between the recognition moieties and the linear
backbone may  lead to mispairing with target bases with
10  two-atom spacers.
        The acylic backbone moiety structures I-N shown
in Figure 4, all of which contain a one-atom methylene
spacer between the backbone and recognition moieties,
gave a good modeling prediction for favorable
15  base-pairing to a complementray polynucleotide.
Analogous structures containing a two-carbon spacer
showed acceptable, but less favorable, binding
conformation.  The structures shown in the figure, and
the analogous two-carbon spacer structures, are
20  preferred acylcic backbone moieties because of their
general ease of synthesis.  It should be mentioned,
however, that a number of other backbone moieties are
also entirely feasible and suitable -- although
generally not as easy and/or inexpensive to synthesize.
25

C.  Backbone/Recognition Moiety Linkage
        As indicated above, the linkage or spacer
connecting the backbone and recognition moieties in the
polymer subunit must meet certain design criteria which
30  are effective to position the recognition moieties for
base-pair binding to the polynucleotide bases.  In the
case of the cyclic backbone moieties shown in Figure 3,
modeling studies indicate that the most favorable

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binding conformation in the polymer occurs  when the
recognition moiety is attached directly to the 1' carbon
of the ribose or ribose-like structures, and to the
analogous 1' position of the morpholino structures.
5   That is, the moiety is attached at normal point of
attachment and with the normal stereoisomeric
configuration of purine or pyrimidine bases to the
ribose or deoxyribose groups of nucleosides.  For the
acyclic structures, one-and two-carbon atom spacers are
required for placing the recognition moieties at
10  favorable binding positions, with one-atom spacers being
preferred, as discussed above.

In order to achieve a stereoregular polymer
configuration required for uniform binding affinity of,
the polymer molecules to a polynucleotide target, it is
15  also necessary that all of the backbone/recognition
moiety linkages be either of define chirality or
achiral.  The linkages are considered to be of defined
chirality, for purposes of definition herein, if each of
the linkages in any given subunit position in all of the
20  polymer molecules has the same stereoisomeric
configuration, or chirality.  That is, although the
subunits in different sequence positions may have
different internal stereoisomer configurations
(including achirality at specific sequence positions),
25  all of the linkages at a given sequence position among
the polymer molecules have the same stereoisomeric
configuration.  Usually defined chirality is most easily
achieved by using the same stereoisomeric configuration
in all of the subunits.
30  For the cyclic backbone moieties, the most
favorable binding occurs in nucleoside analogs having
the natural D-steriosomeric configuration shown in
Figure 3.  Subunits of this type are also readily

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synthesized, as will be discussed below, using natural
D-nucleoside starting material. With reference to
Figure 4, it is seen that only structures I and L have
chiral linkages between the backbone and recognition
moieties. These subunits are readily synthesized in
homochiral form by using homochiral starting material ,
as will be described. For all of the other acyclic
structures shown in Figure 4, the one-atom linkages are
attached to backbone nitrogen atoms, are are therfore
achiral. Also, of course, the methylene spacers are
themselves achiral.


II.  Protective Group Strategies

        Because of the rather high reactivity of the
compounds used for activating and/or coupling the
subunits, it is generally desirable, and often
necessary, to protect the exocyclic ring nitrogens of
the recognition moieties. Selection of these protective
groups is primarily determined by the type of
intersubunit linkage to be used in the polymer assembled
from these subunits, and secondarily determined by the
relative reactivity of the nitrogen to be protected.

        Base-protected nucleosides are also useful
starting reagents in a number of the subunit synthesis
reactions to be described below. Methods for base
protecting a number of the more common ribo- and
deoxynucleosides from Example I are illustrated in
Example 2. The methods detailed in the example are
generally applicable for forming nucleosides with
amine-protective groups.

        When the intersubunit linkage to be used is
relatively stable to nucleophiles, and particularly to
ammonium hydroxide, then the standard base-protective
groups used for nucleic acid chemistry are suitable.

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Such nucleophile-insensitive intersubunit linkages
include carbamate, amide, and sulfonamide linkages.   The
corresponding nucleophile-sensitive protective groups
for the recognition moieties include: benzoyl for the N4
of C; benzoyl or p-nitrobenzoyl for the N6 of A; acetyl
5   or isobutyryl for the N2 of G; and N2,N6-bisisobutyryl
for 2,6-diaminopurine residues.   Removal of these groups
after completion of polymer assembly is effected by
treatment with ammonium hydroxide.

In contrast, when the intersubunit linkage to
10  be used is sensitive to nucleophiles, such as ammonium
hydroxide, suitable protective groups are those which
can be removed by strong non-nucleophilic bases -- via a
β elimination mechanism.   Such nucleophile-sensitive
intersubunit linkages include:   carbonate; ester; and,
15  to a lesser extent, triocarbamate linkages.   Suitable
protective groups for the recognition moieties removable
via β elimination include:   2-(4-nitrophenyl)ethoxy
carbonyl or 2-(phenyl sulfonyl)ethoxycarbonyl for both
the N4 of C and the N6 of A; and the 9-fluorenyl
20  methoxycarbonyl for the N2 of G and the N2 and N6 of
2,6-diaminopurine residues.   Removal of these groups
after completion of polymer assembly is effected by
treatment with the strong nonucleophilic base
1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), under
25  stringently anhydrous conditions.

In regard to temporary protection of a
nucleophile of the backbone moiety (generally N1 in the
above structures), in the general polymer assembly
strategy employs selected backbone-protective groups
30  which are readily cleared by mild acid.   One primary
criterion for selection of protective groups is that
they be adequately stable, but not so stable that
conditions required for their removal would damage the

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growing polymer. A principal problem in the case of
polymers assembled from cyclic backbone moieties is the
particular acid sensitivity of the glycosidic bond
linking protected purine residues to the C1 of their
backbone moieties. A secondary criterion for selection
5   of the backbone protective group is that it be easily
introduced. Based on the above, the following backbone-
protecting groups are preferred: for primary
hydroxyls, the di(p-methoxy)trityl group; for primary
amines, p-methoxytrityl; and for a secondary amine (as
10  in morphilino-type backbone moieties), the
phenylisopropoxycarbonyl group. These protective groups
can be readily removed by treatment with 0.2 M
dichloroacetic acid in dichloromethane.

15

III.   Subunit Synthesis

A.   Cyclic Backbone Moieties
        Subunits having a deoxydeoxynucleoside subunit
20  structure (structure A in Figure 3) can be obtained from
commercial sources or prepared via literature methods,
as described in Example I. The subunits include the
following ribosides and deoxyribosides, which are
identified according to the struture numbers of the
25  recognition moieties given in Figure 1: adenosine and
deoxyadenosine (structure 1); 2,6-diaminopurine riboside
and deoxyriboside (structure 2); cytodine and
deoxycytodine (structure 3); 4-methoxy-2-pyrimidinone
deoxyriboside (structure 4); 2-hydroxy-5-methyl
30  pyrimidine deoxiriboside (structure 5);
2-hydroxypyrimidine riboside (structure 6); guanosine
and deoxyguanosine (structure 7); inosine and
deoxyinosine (structure 8); thioguanosine and

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deoxythioguanosine (structure 9); uradine and deoxyuridine (structure 10); thymidine and 5-methyluridine (structure 11;) and 5-halouridines and 5-halodeoxyuridines (structure 12).

The 5'-amino-2',5'-dideoxyribonucleosides (structure B in Figure 3) are prepared according to methods detailed in Example 3. Briefly, the selected deoxyribonucleosides, base-protected if necessary, are reacted with triphenyl phosphine, carbon tetrabromide, and lithium azide to form the corresponding 5'-azidonucleoside, which is then reduced by hydrogenation in the presence of a palladium on carbon catylyst. The nucleosides may be obtained as in Example 1, and base-protected as in Example 2. The stereochemistry of the reactant nucleosides is preserved in forming the 5' amino nucleoside analogs.

An alternative reduction method is used in reducing the azide group of 5'-azido-5-bromo uridine, as described in Example 3.2. Here non-catalytic hydrogenation is needed to prevent removal of the ring bromine atom. In forming the 5'-amine guanosine compound, the azide is placed on the 5' position by first tosylating the 5' hydroxyl group, then displacing with azide, as detailed in Example 3.2.

The 3'-amino-2',3'-dideoxyribosnucleosides (structure C in Figure 3) are prepared according to methods detailed in Example 4. Briefly, thymidine which is protected at its 5' hydroxyl is tosylated at its 3' hydroxyl, and this is followed by an intramolecular displacement involving the 2 oxy base substituent. The resulting ring is now opened by treatment with azide, yielding a 3' azido analog of the correct stereoisomeric form. This analog can be be converted to a selected-base analog by reacting the azide compound

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with a selected purine or pyrimidine base. The latter
may be base protected, if necessary, for subsequent
subunit coupling reactions, as described below. The
thymidine or other azide analog is then reduced to
produce the desired 3' amine nucleoside. The
5    stereochemistry is of the thymidine starting material is
preserved in the synthesis.

Synthesis of the morpholino-type subunit
derivatives represented by structure D in Figure 3 are
detailed in Example 5 for a variety of different
10   recognition moieties. Briefly, a selected nucleoside,
base-protected if necessary, is dissolved in an ammonium
salt, such as ammonium biborate, and then reacted with
sodium periodate to form transient 2', 3' dialdehydes,
which then close upon an ammonium ion to form morpholino
15   ring having 2' and 4' hydroxyls. The compound is then
treated with sodium cyanoborohydride to remove the ring
hydroxyls. The ring nitrogen is preferably protected as
a 2-phenylisopropylcarbamate for subsequent subunit
coupling. The sterochemistry of the nucleoside starting
20   material is retained.

Deoxyribose subunit structures having a 5'
acetic acid group (structure F in Figure 3) can be
prepared according the general synthetic scheme
described in Example 6, which details the synthesis of
25   the 5' acetic acid compounds represented by structure
F. Here a selected deoxyribonucleoside which is
protected at the 3' hydroxyl is converted to the 5'
aldehyde, and subsequently treated with a 2-carbon
Wittig reagent to give the unsaturated acetic acid
30   derivative. Reduction of the side-chain olefin gives
the desired 5' acetic acid compound. The reaction
preserves the sterochemistry of the starting nucleoside
material

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The analogous 5' formate compound (structure E in Figure 3) is formed by treating the above 5' nucleoside aldehyde with a one-carbon Wittig reagent, and hydroslysing the resulting enol to form form the corresponding formaldehyde derivative, which is oxidized to the desired acid. The reaction preserves the stereochemistry of the starting nucleoside material.

B.  Subunit Synthesis−Acyclic Backbones

The 5-atom chain amino acid subunit represented by Structure I in Figure 4 is prepared according to the general procedures outlined in Examples 7-9. Briefly, an (S)-5 carboxy pyrrolidone was converted to the corresponding stereochemically pure (S)-5-tosylmethyl-2-pyrrolidone by known methods, and then reacted with a selected purine or pyrimidine, to form the corresponding (S)-5-methylpurine or pyrimidine pyrrolidone. The displacement reaction retains the original stereospecificity, so that the subunits which are formed are homoisomeric at the site of attachment of the recognition moiety to the backbone carbon.

The purine used in subunit synthesis is preferably contains an electron-withdrawing group at the 6 position, to reduce the reactivity of the 1 and 3 ring nitrogens, i.e., to minimize pyrrolidone coupling at a ring nitrogen. The pyrrolidone-derivized purine or pyrimidine may then be converted to the amine derivative, and base protected, if necessary, prior to the ring opening step which will be described below. Example 7.1 and 7.2 detail methods for forming the cytosine pyrrolidone and its base-protected derivative. The adenosine pyrrolidone formed in Examples 7.3-7.5 employs 6-chloropurine as the starting material, and the corresponding pyrrolidone is converted

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to the adenosine derivative through azide formation as
described.  The adenosine is base-protected, as in
Example 7.6, prior to ring opening.  A similar approach
is used to generate base protected guanine pyrrolidone,
starting from 2-amino-6 chloropurine, as described in
5   Examples 7.7-7.9.  Similar methods are used in
synthesizing 2,6-diaminopurine pyrrolidone, inosine
pyrrolidone, and 2-hydroxypyrimidine pyrrolidone, also
as detailed in Example 7.

        The pyrrolidone is then treated via a series of
10  reactions which cleave the pyrrolidone ring to  form an
amino acid with a t-BOC protected amine.  Examples 8.1-
8.4 describe the synthesis of such t-BOC amino acids
having one of a number of selected recognition
moieties.  In a final step (Example 5), the t-BOC
15  protective group may be removed to form the
corresponding amino acid represented by structure I in
Figure 4.  The amino acid may be used directly for
subunit coupling, according to reactions given below.
Alternatively, the t-BOC protected compound may be
20  activated at its acid group for use in subunit coupling
reactions.

        The strategy for forming the subunit
represented by structure J in Figure 4 is outlined in
Examples 10.1 and 10.2, for forming the cytodine and
25  uridine analog subunits, respectively.  Briefly, in
forming a pyrimidine type subunit, a 6-member
heterocycle, such as a pyrimidine or nicotinate, is
modified to contain a reactive methylene on the carbon
ring position at which attachment to the backbone is
30  desired.  As detailed in Example 10.1, the cytosine
analog is formed by reacting 5-amino-5-bromopyrimidine
to form the corresponding 5-position carboxaldehyde.  In
Example 12, the uridine analog is formed by converting

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6-hydroxynicotinic esters to the corresponding benzylic alcohols. This compound is converted to the aldehyde by reaction in the presence of manganese dioxide.

The aldehyde compound is then reacted with the desired amino acids, such as 4-aminobutyric acid, with reaction at the amine group producing an unstable imine which is reduced to form the stable amine subunit. The secondary amine can be protected, for subunit coupling involving activation of the acid group, by a tBOC group as described in Example 10.1. The base attachment to the backbone at a backbone nitrogen, through a methylene spacer, insures that the subunits lack chiral centers and so cannot be stereoregular.

To form the subunit structures shown at K in Figure 4, the C-carbon backbone analog of the above compound is prepared, using 3-aminoproprionic acid, and this compound is further treated with nitrous oxide to form the corresponding N-nitroso compound, which is subsequently reduced with $H_2IPd$ in the presence of iron salts to give the hydrazino acid subunit.

II.    Backbone Coupling Reactions

The coupling reactions used in forming the polymer molecules of the invention are stepwise reactions which join one selected subunit or subunit sequence to another selected subunit or subunit sequence. For purposes of the present discussion, the coupling reactions will be described generally with respect to coupling a single subunit $B-R_1$ having a selected recognition moiety $R_1$ to another single subunit $B-R_2$ having the same or a different selected recognition moiety $R_2$, to form a dimer of the form

$$R_1 \quad R_2$$

$$B \quad B \quad ,$$

5      where $R_1$ and $R_2$ have the specific sequence shown.


A.    Subunit Coupling: $N_1$----$N_2$ Backbone
      Configurations

            General methods for coupling subunits having an
10    $N_1$--------$N_2$ backbone configuration are illustrated
      in Figures 5A and 5B.  As will be recalled from Section
      I above, $N_1$ and $N_2$ are nucleophilic backbone groups,
      such as hydroxyl and amine groups, which can be
      activated with an electrophile E, to form an activated
15    $N_1$-E or $N_2$-E backbone group which can then react
      with a second $N_1$----$N_2$ type backbone moiety to form
      an $N_1$----$N_2$-E-$N_1$----$N_2$ backbone-linked dimer.
      The subunit backbones of this types which have been
      described specifically above are the cyclic backbone
      structures A-D in Figure 3.  In structures A and B, the
20    activated $N_2$ nucleophile is the 3' hydroxyl group in
      structure C is the 5' hydroxyl and in structure D, the
      6' position hydroxyl corresponding to the 5' position
      hydroxyl in structure C.
            In a preferred coupling method, which is
25    illustrated in Figure 5A, a subunit having a selected
      recognition moiety $R_1$ is activated with an
      electrophile E.  The star at the recognition moiety
      indicates any required base protection.  As seen in the
      figure, the selected subunit is protected at its $N_1$
30    nucleophile, to ensure that (a) only the $N_2$
      nucleophile is activated and (b) the activated subunit
      cannot self-polymerize.  In structures A, C, and D in
      Figure 3, in which the backbone protection group is on

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the 5' hydroxyl, the protective group is preferably an
acid-labile group, such as dimethoxytrityl (DMTO).

The activating reagent shown in Figure 5A has
the general form:

$$Y\text{--}\overset{X}{\underset{}{C}}\text{--}Y, \text{ where}$$

5

X is oxygen or sulfur, and C is an active electrophile
capable of reacting with a nucleophile, such as a
hydroxyl oxygen or amine nitrogen, with displacement of
Y, to form the activated subunit:

10

Activating agents, such as bis-p-nitrophenyl
carbonate, which give the carbonyl activated subunit
(X=O), are used in forming carbonate and carbamate
subunit linkages.  Similarly, activating agent, such as
thiocarbonyl-di-(1,2,4-triazole) (X=S) are used in
forming thiocarbamate linkages.

15

Subunit activation reactions involving carbonyl
activated subunits are detailed in Example 11 for the
5'-protected, 2' deoxynucleosides represented at A in
Figure 4; in Example 12 for the 5'-protected amino
nucleosides represented at B in Figure 4; and in Example
13 for the N-protected morpholino-type subunits shown at
D in Figure 4.  The general reaction conditions used to
activate the hydroxyl groups in these structures are
generally applicable; subunit activation reactions
involving thiocarbonyl-activated subunits are detailed
in Example 14 for the 5'-amino nucleosides represented
at B; and  in Example 15, for the morpholino-type
subunits shown at D in Figure 3.

20

25

Following the activation reaction, the
activated complex is purified by conventional methods,
such as silica gel chromoatography, and then reacted
with a second subunit whose selected recognition moiety
$R_2$ will form the next in-sequence recognition moiety

30

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in the completed polymer.  The coupling reaction is preferably carried out under mild conditions in which the activated group can react with backbone $N_2$ amine groups, but not $N_1$ hydroxyl groups.  Therefore, the

5 method is suitable for coupling subunits of the type represented by structures B-D in Figure 4, but not subunit structure A.  An advantage of this coupling method is that the second subunit -- which contains an amine $N_1$ nucleophile and a hydroxyl $N_2$ nucleophile

10 -- will be coupled to the first activated subunit only through the $N_1$ amine, so it is not necessary to protect the $N_2$ backbone group.  The resulting dimer subunits are therefore coupled through an $N_2$-E-$N_1$ bond, as indicated in the figure.

15 The oligomer can be extended by repeating the above steps of (a) activating the free $N_2$ nucleophile in the second subunit, separating the activated species from the activating agent, and coupling the activated compound with the next-in-sequence subunit, whose backbone is unprotected.  This method is used

20 particularly in forming short oligomer blocks by solution methods which will be described below and which are suitable for solid-phase block assembly.

For forming a polymer by solid-phase sequential subunit addition, the second coupling method outlined in

25 Figure 5B is much preferred.  The second method differs from the first method in that polymer growth occurs by addition of an excess of activated subunit to an existing subunit or polymer chain, rather than, as in the first method, by addition of an unactivated subunit

30 to an activated chain.  In Figure 5B, the existing subunit or subunit chain is shown by a subunit whose recognition moiety is $R_1$ (second line in Figure 6B).  This subunit has a free $N_1$ backbone nucleophile and an

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$N_2$ nucleophile which is protected by a preferably acid-stable linkage to a solid support or by virtue of being linked to a chain of subunits which are linked to a solid support.  Methods of forming cyclic backbone subunits which are $N_2$ protected in this manner are

5    described generally in Examples 12-15.   The first subunit (the most recently added subunit in a growing polymer) is now reacted with an activated subunit which thereby becomes the next-in-sequence subunit in the polymer.  This activated subunit, which is activated at

10   its $N_2$ backbone site, and protected at its $N_1$ site, preferably by an acid-labile protective group, is prepared by methods described above with reference to Figure 6.

As seen in Figure 5B, the coupling reaction

15   adds the $N_2$-activated second subunit to the $N_1$-protected first subunit to couple the two through an $N_2$-E-$N_1$ bond, and form a compound which is protected at both free backbone nucleophile sites.  This compound is now treated, for example by reaction with

20   acid, to deprotect the acid-labile $N_1$ protective group on last-added subunit, and the procedure is repeated to build up a desired sequence polymer.

It can be appreciated from the above that the $N_2$-activated subunit which will form the

25   last-in-sequence subunit must be protected at its $N_1$ backbone site, to allow selective activation at the $N_2$ site and to prevent  self-polymerization of the activated compound.  Also the $N_1$-deprotected subunit which is to couple to the activated subunit should be

30   protected at its $N_2$ site to allow selective reaction of its $N_1$ moiety with the $N_2$-activated subunit. Therfore, since the reacting subunits are both protected at one backbone site, the method is suitable for

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coupling nucleosides (structure A in Figure 4) as well
as subunits whose cyclic backbones contain both amine
and hydroxyl backbone nucleophiles, such as structures
B-D in this figure.  The reaction methods used in
coupling structure A subunits through a carbonate bond

5   are detailed in Example 12.  Briefly, a subunit
containing a 5' protected backbone moiety is activated
at its 3' hydroxyl, and reacted with another subunit (or
growing chain) which is protected at its 3' hydroxyl.
The coupling reaction is carried out in the presence of

10  a catalyst, such as N-methylimidazole or
N,N-dimethylaminopyridine, which is necessary for
forming the carbonate bond.  For coupling subunits
containing structure B-D cyclic backbones, where
intersubunit carbamate or thiocarbamate bonds are

15  formed, much milder uncatalyzed coupling conditions,
such as those described with reference to the first
method above, are suitable.

        The advantage of this second coupling method,
for forming a polymer by solid-phase subunit addition,

20  is that a substantial molar exess of the activated
subunit can be added to the growing support-bound
polymer at each polymer-addition step, to achieve
subunit coupling to a very high percentage of the
support-bound polymers.  This insures that a large

25  percentage of the support-bound polymers will contain
the complete sequence of subunits desired.  By contrast
in the first method, where the growing polymer is
activated at its last-added subunit, the efficiency of
subunit addition is limited by the efficiency of the

30  activations steps.
        Figure 6 shows the dimeric structures produced
by coupling the cyclic backbone subunits indicated at
A-A through D-D in Figure 4.  The nucleoside subunits in

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structure A in the figure are joined by a carbonate (Y=O). In the remaining three structures B-D, the subunits are joined by carbamate (Y=O) or thiocarbamate (Y=S) bonds. As seen from the figure, all of the subunit linkages are uncharged and achiral, i.e., do not contain chiral centers. In addition, the linkages are stable in aqueous medium, as judged by the ability of polymers to resist hydrolysis over an extended period in neutral aqueous solution.

According to another important feature of the invention, the structures, when joined to form polymers show acceptable Watson/Crick base pairing with complementary polynucleotides.

Finally, according to another important feature of the invention, polymers formed from the subunits are stereoregular. This is achieved in the structures shown by (a) using natural nucleosides or nucleoside derivatives or synthetic nucleosides having the natural stereoisomeric configuration as subunits, and (b) joining the subunits by achiral intersubunit linkages. Exemplary coupling methods are detailed in Examples 11-15.

B.    Subunit Coupling: $N_1$----E Backbone Configurations

General methods for coupling subunits having an $N_1$-----E backbone configuration are illustrated in Figures 7A, 7B, and 7C. As will be recalled from Section I above, $N_1$ is a nucleophilic backbone group, such as a hydroxyl or amine group, and E is an electrophile, such as a carboxyl or sulfonyl group, which, when activated, can react with a second $N_1$-----E type backbone to form an $N_1$-----E-$N_1$ -----E backbone-linked dimer. The subunit backbones of this types which have been described specifically above

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are the cyclic backbone structures E-H in Figure 3, and the acyclic backbone structures I-N in Figure 4. In the cyclic structures E and F, the $N_1$ nucleophile is the 3' hydroxyl group, and in structures G and H, the amine attached to the 3' nitrogen on the morpholino ring. The

5  E group in all of the cyclic structures is the carboxyl or corresponding sulfonyl group attached to the 5' position of the ribose ring, or to the 6' position of the morpholino ring. In all of the structures shown in Figure 4, the $N_1$ and E groups are respectively, the

10 backbone amine or hydrazine and carboxylic or sulfonic acid groups on the backbone moiety ends

The first coupling method, which is illustrated in Figure 7A, is analogous to the method described above with reference to Figure 5A, where a first

15 $N_1$-protected subunit is activated, then coupled directly to a backbone-unprotected subunit which forms the next-in-sequence subunit in the growing polymer. The $P_1$ protective group on the subunit $N_1$ is preferably an acid-labile protective group, such as

20 t-butoxycarbonyl or a cleavable linker bound to a solid support. Methods for $N_1$-protecting cyclic backbone structures are analogous to procedures described above for cyclic structures A-D. Similar methods are effective to protect the amine nitrogens in the acyclic

25 bonebone structures. Alternatively, if the polymer is to be constructed on a solid phase support, as described below, the backbone $N_1$ site may be protected by its attachment to a solid support, with subunit addition to the growing polymer occuring via the activated E

30 electrophile of the last-added subunit.

The activation reaction is designed to yield an activated or chain-terminal moiety of the form $N_1$-----E-X, where X is as described above with

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reference to Figure 6, and the activated subunit has
much the same reactivity toward backbone nucleophilic
groups as does the activated subunit in the previously
described method.  That is, the activation is designed
to produce much the same active coupling group as in the
5   $N_1$----$N_2$ type backbones, but where the reactive
carbonyl or sulfonyl group is provided by the backbone
itself rather than by a carbonyl or sulfonyl activating
reagent, as in the methods shown in Figure 6.

    The activation can be performed readily by
10  reacting the $N_1$----E type subunit with a carbodimide
in the presence of p-nitrophenol, where the backbone
electrophile is a carbonyl group, or by reacting with a
carbodiimide in the presence of imidazole, 1,2,4
triazole or tetrazole, where the backbone is a
15  sulfonyl.  Subunit activation reactions involving
carbonyl electrophilic groups are detailed in Examples
16 and 17.  The general reaction conditions used to
activate the N-amino-morphilino and linear chain
backbone carbonyl groups are generally applicable to the
20  other $N_1$----E type backbone structures shown in
Figures 3 and 4.

    Following the activation reaction, the
activated complex is purified by conventional methods,
such as silica chromatography, or, in the case of a
25  support-bound activated chain, simply washed, and then
reacted with a second subunit whose selected recognition
moiety $R_2$ will form the next in-sequence recognition
moiety in the completed polymer.  The coupling reaction
yields a dimer whose subunits are therefore coupled
30  through an E-$N_1$ bond, as indicated in the figure.  As
above, both subunits must be suitably base protected
during the activation and coupling reactions.

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The polymer can be extended by repeating the above steps of (a) activating the free E electrophile in the second subunit, (b) separating the activated species from the activating agent, and (c) coupling the activated compound with the next-in-sequence subunit, whose backbone is unprotected.

The second coupling method, which is illustrated in Figure 7B, is directly analogous to the method described above with respect to Figure 5B. Here a first subunit having an E-protected backbone is reacted with a second subunit with an activated E group and a protected $N_1$ nucleophile, to form a dimer linked through an $E-N_1$ bond and protected at both free backbone end groups. Where the polymer is being formed by solid-phase synthesis, the $P_2$ protective "group" takes the form of an acid-stable linkage to a solid support. The $N_1$-protected subunit is prepared and activated as above. Coupling conditions generally follow those used in the above cyclic subunit coupling reactions.

In the third coupling method, shown at Figure 7C, the $n_1$-protected and E-unprotected subunits (or polymer units) are reacted together in the presence of a suitable E-activating agent, such as a carbodiimide, as indicated

Figure 8 shows the dimeric structures produced by coupling the cyclic amd acyclic backbone subunits indicated at E-K in Figures 3 and 4, and indicated at E-E through K-K, repsectively. The nucleosides subunits in structure F-F in Figure 3 are joined by an ester linkage. In remaining cyclic backbone structure, G-G, the subunits are joined through a hydrazid or sulfonyl hydrazide linkage. All of the acyclic backbone subunits, shown at I-I, J-J and K-K, are linked through

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amide (E=carbonyl) or sulfonamide (E=sulfonyl) bonds.
As in the above-discussed cyclic backbone subunit
structures, all of the subunit linkages are uncharged
and achiral, and all of the bonds are reasonably stable
in aqueous medium, as judged by the known stability of
ester, hydrazide and amide linkages in solution.

       According to another important feature of the
invention, the structures, when joined to form polymers
show Watson/Crick base pairing with complementary
polynucleotides.

       Finally, as with the $N_1$-----$N_2$ backbone
subunits described above, all of the $N_1$----E backbone
structures shown are stereoregular. This is due to (a)
the homochiral (structures E-H in Figure 3 and I and L
in Figure 4) or achiral (structures J, K, M, and N in
Figure 4) nature of the recognition moiety attachment to
the backbone moiety, and (b) the achiral subunit
linkage.

C.   Subunit Coupling: Alternating Charged and Uncharged
     Achiral Linkages

       In some applications, it may be desirable to
introduce one or more charged achiral subunit linkages
in the polymer molecules. The backbone charge may be
used to enhance polymer solubility in many solution
applications or to adjust polymer-target binding
constants, as will be described further below. Polymers
having regularly spaced charged achiral backbone
linkages, e.g., between every second or third subunit,
are suitable for the novel diagnostic system which is
described below. For purposes of the present
discussion, it is assumed that polymeric units composed
of two or more subunits which are internally joined by
achiral uncharged linkages are to be linked by a charged

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achiral linkage.  These uncharged units are formed by
one of the methods described in Sections A and B above.

The dimer subunits which are to be linked are
selected such that that the free $N_2$ group of one dimer
can be coupled to the free $N_1$ group of the other dimer
5    through a suitable charged achiral linkage.  The
preferred linkage is a phosphodiester linkage which
requires that the free $N_2$ group on one subunit and the
free $N_1$ group on the other subunit both be hydroxyls.
As can be seen in Figure 3, this condition is met if
10   dimers formed from either structure A or structure B
subunits, which both have a free $N_2$ hydroxyl, are
linked to dimers formed from structure A, C, or D
subunits which all have a free $N_1$ hydroxyl.

In a typical coupling method, dimer A, which is
15   protected at its $N_1$ position, as indicated,  is
carried through a series of reactions which lead to an
reactive phosphoester coupling species linked to the
$N_2$ hydroxyl through a phosphoester bond, as indicated
in Figure 9.  The preferred coupling method, which is
20   detailed in U.S. patent application for "Polynucleotide
Assay Reagent and Method", Serial Number 712,396, filed
March 15, 1985, and which follows literature methods for
forming phosphodiester bonds between base-protected
nucleosides or nucleoside analogue subunits,  involves
25   formation of a reactive $N_1$ protected
$N_2$-(p-chlorophenyl2-cyanoethyl)-phosphate.  The
activated dimer is then reacted with the second dimer to
form a tetramer which is linked alternately by uncharged
and charged achiral intersubunit linkages.
30   The phosphodiester linkage method may be
incorporated, either is solution or support-type polymer
synthesis method, according to the general procedures

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outlined above for polymer coupling involving achiral,
but uncharged linkage reactions.

Although for diagnostic purposes, polymers with
alternating charged and uncharged achiral bonds may be
5   suitable, it is generally preferred in diagnostic and
particularly therapeutic uses, to introduce as few
charged linkages as are necessary for achieving adequate
polymer solubility in an aqueous medium.  The solubility
enhancement is generally achieved with 1-3 charged
backbone linkages per polymer of about 20 subunits, and
10  preferably no more than about one charged linkage per
four subunits.  As defined herein, achiral backbone
linkages are considered substantially uncharged if they
contain less than about one charge backbone linkage per
dimeric unit, and preferably one or fewer backbone
15  linkage per tetrameric unit.


IV.   Polymer Targeting Considerations


A.   Diagnostic Applications
20
An important application of the uncharged or
substantially uncharged polymers of the invention is in
a novel solid-support diagnostic system for use in
determining the presence and/or amount of a
polynucleotide analyte.  The assay system includes a
25  solid support reagent composed of a multiple
polynucleotide-binding polymers which are constructed
according to the invention linked to a solid support.
The polymers are constructed, according to targeting
strategies detailed below, for binding specifically to
30  target polynucleotide analyte at a selected melting
temperature ($T_m$).  The sytstem also includes
polycationic reporter molecules which are designed to
bind to the fully charged analyte backbone, but not the

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uncharged or only partially charged polymer backbone,
under selected binding conditions.  Each reporter
molecules carries one or more reporter groups, and each
polynucleotide can accomodate binding of typically
several thousand or moe reporter molecules.  Thus the
5    system has an ampliication factor, in terms of reporter
signal per bound analyte molecule, of several orders or
magnitude.  The assay system and its method of practice
are more fully described in co-owned U.S. Patent
application for "Polynucleotide Assay Reagent and
10   Method" , Serial No. 712,396, filed March 15, 1985.
        The particular merits of using
sequence-specific polynucleotide binding polymers having
achiral predominatly uncharges intersubunit linkages for
diagnostic applications derives from two factors.
15   First, their target binding is relatively insensitive to
salt and so the polymer/target annealing step can be
carried out under low salt conditions wherein there is
no competitive annealing between the target and
complementary nucleic acid sequences in the solution.
20   Second, the polymer's reduced charge or uncharged
backbone allows use of the above-described reporter
molecules which are designed at high density to the
target analyte's polanionic backbone.
        The design considerations applied in preparing
25   a polynucleotide binding polymer for use as a diagnostic
reagent are governed by the nature of the target analyte
and the reaction conditions under which the analyte is
to be assayed.  As a first consideration, there is
selected a non-homopolymeric target base sequence
30   against which the polymer is directed.  This target
sequence is preferably unique to the analyte being
assayed, i.e., is calculated to occur only with some
defined small probability (such as 1% or less) in an

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assay mixture containing a given number of unique-sequence bases. The probability of occurrence of a given n-base target sequence is approximately $1/4^n$ — that is, a given n-base target sequence would be expected to occur approximately once in a polymer

5   containing $4^n$ bases. Therefore, the probability P that a given n-base sequence will occur in polynucleotides containing a total of N unique-sequence bases is approximately $P=N/4^n$. To illustrate, the probability P that a 9-base target sequence will be found in a 20

10   kilobase polynucleotide is about $20 \times 10^3 / 2 \times 10^5$ or 0.08, the probability that a 16-base target sequence will be present is about $20 \times 10^3 / 4.3 \times 10^9$ or 0.0000047. From these calculations, it can be seen that a polymer having 9-16 recognition moieties specific for

15   a defined 9-16 base target sequence should have high specificity for the target sequence in an assay mixture containing only viral genomes, whose greatest complexities correspond to about 400K of unique-sequence bases.

Similar types of calculations show that a 12 to

20   16 subunit polymer can provide adequate specificity for a viral or bacterial target sequence in an assay mixture containing viral and bacterial genomic material only (largest genomic sizes about 5,000 kilobases), and that a 16 to 22 subunit polymer can provide adequate

25   specificity for a target sequence in a polynucleotide mixture containing mammalian genomic DNA material (genomic sizes of about 5 billion base pairs of unique-sequence DNA).

The polymer/analyte binding affinity, and

30   particularly the temperature at which the polymer just binds with the target sequence (the melting temperature, or Tm) can be selectively varied according to (a) polymer length, (b) the number of hydrogen bonds that

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can be formed between the recognition moieties and the
corresponding, in-sequence bases of the analyte target
sequence, (c) backbone charge density, and (d) a
concentration of denaturants, such as formamide, which
reduces the temperature of melting.  From a number of
5   studies on model homopolymer duplexes, it is known that
the melting temperature of oligonucleotide duplexes in
the 10 to 20 bp range increases roughly 3°C per
additional base pair where the complementary bases are
capable of forming two hydrogen bonds, and about 6°C per
10  additional base pair where the complementary bases are
capable of forming three hydrogen bonds.  Therefore, the
length of a target sequence, which is initially selected
to insure high binding specificity with the polymer, may
be extended to achieve a desired melting temperature
15  with the complementary-base polymer, under selected
assay conditions.  Also, where the recognition moieties
used in constructing the polymer are the standard
nucleic acid bases, as illustrated above, the target
sequence may be selected to have a high percentage of
20  guanine plus cytosine bases for achieving a relatively
high polymer/analyte melting temperature, or a
relatively high percentage of adenine plus thymine
bases, for achieving a relatively low melting
temperature.
25       The backbone charge density of the polymer must
be substantially less than that of the polynucleotide
analyte, to allow preferential binding of polycationic
reporter molecules to the analyte, under conditions
where reporter binding to the polymer does not occur.
30  This requirement is met where the spacing between the
adjacent negative charges in the polymer backbone is at
least twice as great as the spacing between adjacent
charged phosphodiester linkages in the analyte.  The

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charge density of the backbone also has an important
effect on the polymer/analyte melting temperature,
particularly under low salt conditions, where any
charge-charge repulsion between the analyte and polymer
backbones can act to lower the temperature at which the

5     two can anneal.  In general, therefore, a polymer having
a less-charged backbone would be expected to show (1) a
higher analyte/polymer melting temperature, and (2) less
dependence of the melting temperature on salt
concentration.  Based on these considerations, an

10    uncharged polymer, such as an all-carbamate-linked or
all amide-linked polymer, will allow the analyte/polymer
annealing reaction in the diagnostic method to be
carried out under a wider range of salt-concentrations
than a partially charged polymer, wherein some of the

15    linkages constitute achiral but charged phosphodiester
linkages.


B.   Therapeutic Applications

20 
Requirement for Adequate Specificity


1.   Information Requirements

       An ideal therapeutic is one which efficiently
inactivates a targeted structure unique to the

25    pathogenic state, but which does not detrimentally
affect normal structures in the patient.  Thus,
sequence-specific gene inactivating agents should only
effect inactivation of a targeted genetic sequence which
contains an amount of sequence information adequate to

30    assure a high probability of that target sequence being
unique to the pathogenic state.  For example, an agent
which can effect target inactivation by binding to only
three or four contiguous bases of its target sequence

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would invariably also attack many mRNAs required for normal cellular functions. In contrast, an agent which requires binding to 30 or more bases in order to effect target inactivation would have an exceedingly small probability of also attacking non-target messengers involved in normal cellular functions.

A reasonable estimation of the desired amount of sequence information which a gene-inactivating agent should recognize when that agent is targeted against a disease-specific mRNA can be calculated as follows. The human genome contains roughly 5 billion base-pairs of unique-sequence DNA. However, essentially all of this material exists in the duplex state and so is largely unavailable for binding to single-strand-directed polynucleotide-binding agent. Thus, polymer binding is largely restricted to RNA transcripts of the genomic DNA. Of that genomic DNA, something on the order of 1% is transcribed in any given cell type. Further, when all cell types in the patient are considered, it is unlikely that a composite of more than 4% of the entire genome is transcribed in the adult human (i.e., post-embryogenesis). Thus, the sequence complexity of all rna sequences inthe adult human is likely to constitute no more than about 200 million bases -- and probably far fewer.

For a gene-inactivating agent to have an expectation of recognizing no target sequences in a cellular pool of 200 million bases of unique sequence genetic material, it should recognize at least n bases in its target, where n is calculated as $2 \times 10^8 = 4^n$ -- giving a minimal target recognition requirement of 14 bases. This suggests that a gene-inactivating agent recognizing in excessof 14 bases in its target will likely have no targets in cellular pools of normal RNA.

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Obviously, as the number ob bases recognized in the
target sequence increases over this value, the
probability that the agent will recognize no inherent
cellular RNA sequences also increases.  It is noteworthy

5  that as the number of bases recognized in the target
increases linearly, this "safety factor" increases
exponentially.  For example, below are tabulated the
number of bases recognized in a target sequence and the
corresponding expected number of targets in a pool of
200 million bases of unique-sequence single-stranded

10 genetic material:

| No. of bases recognized | Expected no. of targets |
|-------------------------|-------------------------|
| 12                      | 12.0                    |
| 14                      | .75                     |
| 16                      | .047                    |
| 18                      | .0029                   |
| 20                      | .00018                  |

20      From the above one can conclude that for a
sequence-specific gene-inactivating agent destined for
therapeutic applications, safety considerations probably
mandate that the agent recognize at least 14, and
preferably 16 to 20, bases in its target sequence.

25

2.   The Concept of a Minimum Inactivation Length (MIL)
In regard to the amount of sequence information
actually "recognized" by a sequence-specific gene-inac-
tivating agent, a sueful concept in this regard is that
of a minimum inactivating length (MIL), which we define

30 as the minimum number of contiguous bases of a target
sequence to which an agent must bind in order to effect
gene inactivation in vivo.  Irrespective of whether or
not the agnet binds a longer sequence of bases, it is

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this MIL value which determines the number of bases in the targer "recognized" by that agent -- which, in turn, determines the agent's level of sequence specificity.

To illustrate this concept, suppose a gene-inactivating agent has a 16-subunit length but an MIL value of only 10. ·Such an agent would be expected to inactivate any genetic sequences containing any of the component 10-contiguous-base target sequences. In a cellular pool comprising 200 million bases of unique-sequence single-stranded genetic material, there are an estimated 1000 such target sequences. Further, because the number of base-pairing moieties in the agent is substantially greater than the MIL value, the agent would also be expected to forma variety of partially mispaired complexes with sequences lacking even the required 10-contiguous-base target sequences -- with some of those mispaired complexes having sufficient stability to effect inactivation of the participating nontarget genetic sequences.

In contrast, in the case of an agent 16 subunits in length and having an MIL value of 16, by definition, such an agent cannot inactivate nontarget sequences because any partially mispaired complexes with nontarget sequences would not have sufficient stability to effect gene inactivation -- only perfect 16-base-pair agent/target duplexes would have adequate stability to effect gene inactivation.

In regard to determining actual MIL values, when a series of gene-inactivation agents, all targeted against the same genetic sequence but varying in number of subunits, are tested in living cells known to contain a functioning single-stranded target sequence, the activity of the targeted gene will show a dramatic reduction at the subunit length corresponding to the MIL

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value.   That is, the MIL value corresponds to that
number of subunits just sufficient to block the activity
of the targeted genetic sequence.

        To illustrate further, below is described an
5  exemplary method for determining the minimum
inactivating length for a polynucleotide-binding polymer
having any selected backbone linkage type.

        First, prepare a regular series of binding
polymers having the selected backbone type, targeted
10 against the region 57 to 80 of the rabbit β-globin in
mRNA (5'-GUGCAUCUGUCCAGTGAGGAGAAG-3'), and ranging in
length from 5 to 24 subunits.   Next, following the
detailed procedures of Black, Murakimi, and Miller,
Biochemistry, 24:6132 (1985), add each of these polymers
15 to a separate portion of a commercial rabbit
reticulocyte lysate preparation labeled with
[$^{35}$S]-methionine.   Thereafter, add rabbit globin mRNA
and incubate 1 hour at 37°C.   Work each sample up,
fractionate by gel electrophoresis, and quantitate the
20 radioactivity incorporated in β-globin as per Blake et
al, above.   The minimum inactivating length is that
minimum polymer subunit length which effects essentially
complete blockage of new β-globin synthesis.

        It should be reemphasized that this MIL will
25 vary at small amount depending on the targeted sequence,
but in general this relatively simple extracellular
method serves to give a quite good approximation of the
intracellular MIL for any given backbone type.   Once
this MIL value has been determined for a selected
30 backbone type, the methods described below for MIL
adjustment can be rationally applied for optimizing the
MIL for a selected target sequence.

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## 3. Tactics for Adjusting the MIL

As a rule, it is often necessary to vary an agent's target-binding affinity over a substantial range in order to ultimately achieve an acceptable MIL value. To this end, a variety of methods, and combinations thereof, can be used for varying the stability of the agent/target duplex, including:

(i) increasing or decreasing the number of base-pairing moieties in the agent;

(ii) selecting a target having a sequence providing greater or lesser stacking contributions (see Tnoco et al (31) for a detailed analysis of the effects of base sequence on duplex stability);

(iii) selecting a target sequence containing a greater or lesser proportion of strong-binding bases (G and C) to weak-binding bases (A and U or T);

(iv) replacing one or more weak-binding base-pairing moieties with stronger-binding moieties having the same Watson/Crick pairing specificities (e.g., a 2,6-diaminopurine moiety can be used instead of adenine to effect a substantially stronger bond to U or T in the target sequence; a 5-bromouracil moiety can be used instead of U or T to effect a substantially stronger bond to a in the target sequence; and a 5-bromocytosine moiety can be used instead of cytosine to effect a somewhat stronger bond to G in the target sequence); or replacing one or more strong-binding base-pairing moieties of the agent with a weaker-binding moiety having the same W/C pairing specificities (e.g., a 2-pyrimidinone moiety can be used instead of cytosine to effect a substantially weaker bond to G in the target sequence; and a 6-thioguanine or hypoxanthine moiety can be used instead of guanine to effect a substantially weaker bond to C in the target sequence); and

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     (v)   selecting a backbone structure so as to somewhat inhibit or enhance Watson/Crick pairing to the target sequence.

5      It should be noted that the first three of the above methods actually comprise target selection procedures, while the latter two methods are effected during synthesis of the agents.

     A difficulty with method (i) is that sequence specificity requirement generally preclude reduction of

10 the agent's length to less than about 14 subunits.  The difficulty with methods (ii) and (iii) is that other more critical targeting criteria can so severely limit the optimal target region that these two MIL adjustment methods have little latitude in which to achieve a

15 significant effect.  Because of these limitations, most MIL adjustments are preferably achieved by methods (iv) and (v).


V.  Polymer Assembly Strategies

20

A.  Geometric assembly

     After selecting a desired polymer length and recognition moiety sequence, according to factors considered in Section IV, the polymer is assembled,

25 using the general subunit coupling procedures detailed above.  One method of polymer assembly involves initial preparation of an appropriate set of dimers, linking selected dimers to form tetramers, linking of these to form octamers, and so on.  This method is carried out in

30 solution, substantially according to methods described with reference to Figures 5A and 7A above.  It should be noted that all couplings need not necessarily be between oligomers of equal size.  For example, often it is desirable in the last coupling to link a hexadecamer

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with a tetramer to give a 20-mer or to link a
hexadecamer with an octomer to give a 24-mer.

A particular merit of this assembly method is
that each coupling product is roughly twice the mass of
5   the precursors and so purification of the product of
each coupling is a simplified matter.  Example 18 below
details the the assembly of an 8-subunit
carbamate-linked polymer formed by this method.(b)

10  B.  Stepwise Assembly on a Solid Support

One preferred method for polymer synthesis is
stepwise assembly on a solid support.  Here the first
subunit in the polymer is attached through its $N_2$
backbone group to a solid support.  Typically, a solid
15  support, such as glass beads derivatized with cleavable,
preferably acid-stable, long-chain linkers are employed
as the support material, and prepared for attachment of
$N_2$ nucleophile, such as a 5' hydroxyl of a nucleoside,
as descibed in Example 19.  The glass beads are reacted
20  with a subunit which has a preferably acid-labile $N_1$
protected group, and an activated $N_2$ or E backbone
group, as detailed in Section III above.  The coupling
of various types of subunits to a glass-bead support is
described generally in Examples 20-22.

25  After coupling the second subunit (or polymer
unit which may be assembled in solution) to the support,
any unreacted linker nucleophiles are capped by addition
of a suitable capping reagent, such as p-nitrophenyl
acetate, and thereafter the support is washed and
30  filtered.  The protecting group on the $N_1$ terminal
subunit is removed, typically by acid treatment, and
after neutralization, the support is then reacted with
an excess of the next-in-sequence subunit (or polymer
unit) which is activated at its free $N_2$ backbone

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moiety.  The excess of activated subunit maximizes the
number of support-bound subunits which are
chain-elongated.  That is, one feature of the solid
support assembly method is the need for high coupling
5    efficiencies at each subunit addition step, and the
concentration of added activated subunit is selected to
maximize this efficiency.  Chain elongation is continued
in this manner, with capping of failure sequence, after
each subunit addition, until the polymer of the desired
10   length and sequence is achieved.  The general method is
illustrated in Example 20 for the preparation of a
14-subunit carbonate-linked polymer, in Example 21, for
the synthesis of a 19-subunit carbamate-linked polymer,
and in Example 22, for the assembly of a 19-subunit
15   amide-linked polymer.

   After addition of the last-in-sequence subunit,
the polymer may be capped with a suitable charged or
uncharged group coupled to the terminal $N_1$ group.  If
the backbone assembly has been carried out exclusively
20   with uncharged achiral linkages, the capping reagent is
preferably a charged group which imparts an end terminal
charge to the polymer.  After capping the polymer, the
polymer is cleaved from the support, e.g., by treatment
with a nucleophile, such as ammonium hydroxide, and the
25   polymer is purified, such as by anion exchange
chromatography.  These post-assembly operations are
described in Example 23, for synthesis of a
carbonate-linked polymer, in Example 25, for synthesis
of a carbamate -linked polymer, and in Example 27, for
30   synthesis of an amide-linked polymer.

   For diagnostic applications involving the above
solid-support reagent with bound polymer molecules, the
assembled, purified polymers are attached to a solid
support, such as a cellulose support by known coupling

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methods, such as those detailed in Example 20. Examples 24, 26, and 28 are illustrative.

The following examples illustrate a variety of subunit synthetic schemes, polymer construction methods, and applications and compositions made and used according to the invention. The examples are in no way intended to limit the scope of the invention.

## Example 1
### Preparation of ribonucleosides and deoxyribonucleosides

The following nucleosides are obtained from Sigma Chemical Co., St. Louis, MO: deoxyuridine, deoxyguanosine, thymidine, deoxyadenosine, deoxycytidine, 5-bromodeoxyuridine, deoxyinosine, 2,6-diamino-9-(2-deoxy-β-D-erythro-pentofuranosyl)9H-purine (2,6-diaminopurine deoxyriboside), uridine, guanosine, 5-methyluridine, adenosine, cytidine, 5-bromouridine, inosine.

2,6-Diamino-9-(β-D-ribofuranosyl)-9H-purine (2,6-diaminopurine riboside) is obtained from Pfaltz and Bauer, Inc., Division of Aceto Chemical Co., Inc., Waterbury, CT.

The following nucleosides are prepared by the literature methods indicated:

1-(2-Deoxy-β-D-erythro-pentofuranosyl)-2-pyrimidinone (2-hydroxypyrimidine deoxyriboside) is prepared by the method of P. Kohler, E. Volz, U. Sequin, and C. Tamm in Nucleic Acid Chemistry (L.B. Townsend and R.S. Tipson, eds) John Wiley and Sons, Inc. (1976).

1-(2-Deoxy-β-D-erythro-pentofuranosyl)-4-methoxy-2-pyrimidinone is prepared by the following procedure:

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1-(3',5'-Di-O-benzoyl-2-deoxy-ß-D-erythro-pentofu
ranosyl)-4-methylthio-2-pyrimidinone (prepared as in D.
Cech and A. Holy, <u>Collection of Czechoslov Chem Comm</u>
(1977) <u>42</u>:2246) is treated with 200 mL of 0.2M sodium
5  methoxide solution.  The resulting solution is allowed
to stand overnight at room temperature.  This solution
is then neutralized with Dowex 50X8 (H+ form) and
filtered; the residue is dissolved in water.(100 mL)
extracted with ether (2.50 mL) and the aqueous phase
10 evaporated.  The residue is an amorphous material which
isused directly in succeeding reactions.
2-Amino-9-(2-deoxy-ß-D-erythro-pentofuranosyl)-1,
9-dihydro-6H-purine-6-thione (deoxythioguanosine) is
prepared by the procedure of R.H. Iwamoto, E.M. Acton,
15 and L. Goodman, <u>J Med Chem</u> (1963) <u>6</u>:684.
1-(ß-D-Ribofuranosyl)-2-pyrimidinone
(2-hydroxypyrimidine riboside) is prepared by the
procedure of U. Niedballa and H. Vorbruggen, <u>J Org Chem</u>
(1974) <u>39</u>:3668.
20         1-(2-Deoxy-ß-D-ribofuranosyl)-4-methoxy-2-pyrimid
inone (2-hydroxypyrimidine deoxyriboside) is prepared by
the procedure of R. Wightman and D. Holy, <u>Collection of
Czechoslov Chem Comm</u> (1973) <u>38</u>:1381.
2-Amino-9-(ß-D-ribofuranosyl)-1,6-dihydro-
25 6H-purine-6-thione (thioguanosine) is prepared by the
procedure of J.J. Fox, I. Wempen, A. Hampton and I.L.
Doerr, <u>J Amer Chem Soc</u> (1958) <u>80</u>:1669.

### Example 2
### Preparation of Base-Protected Nucleotides
30     Dimethoxytrityl chloride, N-benzoyladenosine,
N-benzoyl-2'-deoxyadenosine, N-benzoylcytidine,
N-benzoyl-2'-deoxycytidine and
N-isobutyryl-2'-deoxyguanosine are obtained from Sigma

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Chemicals, St. Louis, Missouri.
9-Fluorenylmethoxycarbonyl chloride (FMOC chloride),
trimethylchlorosilane, isobutyric anhydride,
4-nitrobenzoyl chloride and all organic solvents for
5  reactions and chromatography are obtained from Aldrich
Chemical Co., Milwaukee, Wisconsin. Silica Gel is
obtained from EM Science, Cherry Hill, New Jersey.

2.1 <u>Guanosine</u>

10      The N-2 9-fluorenylmethoxycarbonyl derivative
is prepared by the procedure below which is general for
the protection of nucleoside amino groups:

     Guanosine (1 mmol) is suspended in pyridine (5
mL) and treated with trimethylchlorosilane (5 mmol).
15  After the solution is stirred for 15 minutes
9-fluorenylmethoxycarbonyl chloride (5 mmol) is added
and the solution maintained at room temperature for 3
hours. The reaction is cooled in an ice bath and water
(1 mL) is added. After stirring for 5 minutes conc.
20  ammonia (1 mL) is added and the reaction stirred for 15
minutes. The solution is evaporated to near dryness and
the residue dissolved in chloroform (10 mL). This
solution is washed with sodium bicarbonate solution (5
mL, 10%), dried over sodium sulfate and evaporated. The
25  residue is coevaporated several times with toluene and
the product chromatographed on silica gel using a
gradient of methanol in methylene chloride (0-50%).

     N-Isobutyrylguanosine is prepared by the method
of Letsinger and Miller, <u>J Amer Chem Soc</u> (1969)
30  <u>91</u>:3356.

     N-2 Acetylguanosine is obtained by the method
of C.B. Reese and R.S. Saffhill, <u>J Chem Soc Perkin Trans</u>
(1972) <u>1</u>:2937.

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2.2 <u>Deoxyguanosine</u>

The N-2 9-fluorenylmethoxycarbonyl derivative
is prepared by the method of J. Heikkla and J.
Chattopadhyaya, <u>Acta Chem Scand</u> (1983) <u>B37</u>:263.

5       The N-2 acetyl derivative is obtained from
Research Plus Inc., Bayonne, N.J.


2.3 <u>Deoxyadenosine</u>

The N-6 2-(4-nitrophenyl)-ethoxycarbonyl

10  derivative is prepared by the method of F. Himmelsbach
and W. Pfleiderer, <u>Tetrahedron Lett</u> (1983) <u>24</u>:3583.

N-6 4-Nitrobenzoyl-2'-deoxyadenosine is
prepared using the procedure above for FMOC-guanosine
except that 4-nitrobenzoyl chloride is substituted for

15  FMOC chloride.

The N-6 2-(phenylsulfonyl)-ethoxycarbonyl
derivative is prepared by the procedure for FMOC
guanosine except the 2-(phenylsulfonyl)-ethyl
chloroformate (obtained from N. Balgobin, S. Josephson

20  and B. Chattopadhyaya, <u>Tetrahedron Lett</u> (1981) <u>22</u>: 3667)
is used as the acylating agent and N-methylimidazole or
pyridine is used as the solvent.


2.4 <u>Adenosine</u>

25      The N-6 2-(4-nitrophenyl)-ethoxycarbonyl
derivative is prepared by the method of F. Himmelsbach
and W. Pfleiderer, <u>Tetrahedron Lett</u> (1983) <u>24</u>:3583.

N-6 4-Nitrobenzoyladenosine is prepared using
the procedure above for FMOC-guanosine except that

30  4-nitrobenzoyl chloride is substituted for FMOC
chloride.

The N-6 2-(phenylsulfonyl)-ethoxycarbonyl
derivative is prepared by the procedure for FMOC
guanosine except the 2-(phenylsulfonyl)-ethyl

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chloroformate (obtained from N. Balgobin, S. Josephson
and B. Chattopadhyaya <u>Tetrahedron Lett</u> (1981) <u>22</u>:3667)
is used as the acylating agent and N—methylimidazole or
pyridine is used as the solvent.

5

2.5 <u>Deoxycytidine</u>

The N-4 2-(4-nitrophenyl)-ethoxycarbonyl
derivative is prepared by the method of F. Himmelsbach
and W. Pfleiderer, <u>Tetrahedron Lett</u> (1983) <u>24</u>:3583.

10      The N-6 2-(phenylsulfonyl)-ethoxycarbonyl
derivative is.prepared by the procedure for FMOC
guanosine except the 2-(phenylsulfonyl)-ethyl
chloroformate (obtained from N. Balgobin, S. Josephson
and B. Chattopadhyaya <u>Tetrahedron Lett</u> (1981) <u>22</u>:3667)
15  is used as the acylating agent and N—methylimidazole or
pyridine is used as the solvent.

2.6 <u>Cytidine</u>

The N-4 2-(4-nitrophenyl)-ethoxycarbonyl
20  derivative is prepared by the method of F. Himmelsbach
and W. Pfleiderer, <u>Tetrahedron Lett</u> (1983) <u>24</u>:3583.
The N-6 2-(phenylsulfonyl)-ethoxycarbonyl
derivative is prepared by the procedure for FMOC
guanosine except the 2-(phenylsulfonyl)-ethyl
25  chloroformate (obtained from N. Balgobin, S. Josephson
and B. Chattopadhyaya <u>Tetrahedron Lett</u> (1981) <u>22</u>:3667)
is used as the acylating agent and N—methylimidazole or
pyridine is used as the solvent.

30  2.7 <u>2,6-diaminopurine riboside</u>

The N-2,N-6-bis(9-fluorenylmethoxycarbonyl)
derivative of 2,6-diaminopurine riboside is prepared by
the general procedure.

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The N-2,N-6-bis(isobutyryl) derivative is prepared by the general procedure.

## 2.8 2,6-diaminopurine-2'-deoxyriboside

The bis N-2,N-6-(9-fluorenylmethoxycarbonyl) derivative of 2,6-diaminopurine-2'-deoxyriboside is prepared by the general procedure.

## 2.9 Thioguanosine

The N-2 9-fluorenylmethoxycarbonyl derivative of thioguanosine is prepared by the general procedure.

## 2.10 2'-Deoxythioguanosine

The N-2 9-fluorenylmethoxycarbonyl derivative of 2'-deoxythioguanosine is prepared by the general procedure.

## Example 3

### Preparation of 5'-amino-2',5-dideoxyribonucleoside subunits.

Carbon tetrabromide, sodium azide, p-toluenesulfonyl chloride (tosyl chloride), triphenyl phosphine and 10% palladium on carbon are purchased from Aldrich Chem Co. Lithium azide is obtained from Kodak Laboratory and Specialty Chemicals.

### 3.1 General Method

The nucleosides employed in this example are thymidine, N6-benzoyl-2'-deoxyadenosine, N4-benzoyl-2'-deoxycytodine, and 2'-deoxyinosine, 2-hydroxy-pyrimidine-2'-deoxyriboside and the N2-N6-bisisobutyryl derivative of 2,6-diamino purine-2'-deoxyriboside (see Example 1). The required dried nucleoside (1 mmol) is weighed into a reaction

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vessel containing triphenyl phosphine (1.01 mmol) and
lithium azide (5 mmol). After the solids are suspended
in DMF, carbon tetrabromide (1.0 mmol) is added to the
vessel. The solution is stirred at room temperature for
5  24 h. After quenching the reaction with methanol, the
solution is evaporated to dryness. The residue is
chromatographed on silica gel eluting with methanol/
chloroform mixtures to afford the desired 5'-azido-2',
5'-deoxynucleoside.

10      The 5'-azido nucleoside (1 mmol) is dissolved
in ethanol. Hydrogenation in the presence of 10%
palladium on carbon (catalytic amount) under a hydrogen
atmosphere (35 psi) occurs over 10 h. The solution is
filtered and evaporation under reduced pressure affords
15  a crude solid. The solid is purified by trituration
with the appropriate solvent.

3.2  5'-Azidouridine derivativ

        The 5'-azido derivative of 5-bromo-2'-deoxy
uridine is prepared via the method described above. The
20  5'-azido-t-bromo-2'-deoxyuridine (1 mmol) is treated
with triphenyl phosphine (1.5 mmol) in pyridine at room
temperature for 2 h. Concentrated ammonia (1 mL) is
added to the reaction vessel. After 14 h the solvent is
removed under vacuum. The residue is dissolved in THF
25  and this solution is added to hexanes. The precipitate
is collected and the solid triturated with the
appropriate solvent to afford the 5'-amino-5-bromo-
2'-deoxyuridine.


30  3.5  5'-Azido guanosine

        An alternate preparation of
5'-amino-2',5'-dideoxyguanosine is to treat the
protected 2'-deoxyguanosine (protected at either the
N-2-acetyl or the N-2-FMOC derivative) (1 mmol) with

tosyl chloride (1.3 mmol) in pyridine at 0°C overnight.
The solution is evaporated to dryness and the residue is
dissolved in chloroform.  The resulting solution is
washed twice with aqueous sodium bicarbonate and dried
5   over sodium sulfate.  The solvent is evaporated and the
residue chromatographed on silica gel eluting with
methanol/chloroform mixtures.  The resulting tosylate (1
mmol) is treated with sodium azide (6 mmol) in DMF at
80-100°C for a few h.  The solvent is removed by rotovap
10  and the residue is chromatographed on silica gel eluting
with chloroform/methanol solvent mixtures.  The azide is
reduced to the desired amine using the above procedure.
        Alternate protecting groups for the base
nitrogens of 2'-deoxycytidine, 2'-deoxyadenosine,
15  2'-deoxyguanosine, and 2,6-diaminopurinedeoxyriboside
are 2-(phenylsulfonyl)ethoxycarbonyl for
2'-deoxycytidine and 2'-deoxyadenosine and the use of
the 9'-fluorenylmethoxycarbonyl (FMOC) to protect
2'-deoxyguanosine and 2,6-diaminopurine deoxyriboside,
20  as in Example 2.


## Example 4
### Preparation of 3'-amino-2',3'-dideoxyribonucleoside subunits.

25          Thymidine is transformed to 5'-O-acetyl-3'-
azido-2',3'-dideoxythymidine and this is converted to
3'-azido-2',3'-dideoxyadenosine and 3'-azido-2',3'-
dideoxyguanosine via the methods of M. Imazawa and F.
Eckstein, J Org Chem (1978) 43:3044.  The base moiety of
30  the 3'-azidoadenosine is protected as either the benzoyl
or the 2-(phenylsulfonyl)ethoxycarbonyl derivative (as
shown in Example 1).  N-2 of the 3'-azidoguanosine is
protected as either the acetyl or FMOC derivative (see
Example 1).  The reduction of these 3'-azido functions

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to the 3'-amino-2',3'-dideoxyribonucleosides is
performed as shown in Example 3.

### Example 5

5  Preparation of morpholino-type subunits derived from
ribonucleosides.

Ammonium biborate, sodium m-periodate, sodium
cyanoborohydride, uridine, N4-benzoyl cytidine, and
N6-benzoyl adenosine are obtained from Sigma Chemical

10  Co. N2-isobutyryl guanosine is prepared by the general
method of Letsinger and Miller (J Amer Chem Soc (1969)
91:3356). 2-Phenyl-2-propanol and bis(p-nitrophenyl)
carbonate are obtained from Aldrich Chemical Co.

The morpholino derivative of uridine is

15  prepared by dissolving 1 mmol of uridine plus 2 mmol of
ammonium biborate, $(NH_4)_2B_2O_7$, in 5 ml of
water. While stirring in an ice bath protected from
direct light 1.2 mmol of sodium m-periodate in 5 ml of
water is added. After 90 min, 0.2 ml of 1,2-propanediol

20  is added and stirring is continued for 10 min at rt.
Thereafter, while stirring in a well-ventilated hood,
0.25 g of sodium cyanoborohydride dissolved in 3 ml of
water is added and the mixture stirred for 4 hr at rt.
Thereafter, the reaction mixture is reduced to an oil

25  under vacuum in a warm water bath, resuspended in a
minimal volume of methanol, layered on a silica gel
chromatography column, and the column eluted with
methanol/1% triethylamine. The morpholino product
elutes from the column as a sharp band moving

30  significantly slower than the original ribonucleoside
and its oxidation products. The morpholino product has
a UV spectrum essentially identical to the parent
ribonucleoside, but its mass is lower by 17 daltons

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(determined by mass spectral analysis using fast atom
bombardment activation).

The morpholino nitrogen is next protected by
reacting the product with 2-phenylisopropyl phenyl
carbonate.  The required active carbonate is prepared
and reacted with the morpholino subunit essentially by
the methods of Sandberg and Ragmarsson (Int J Peptide
Protein Res (1974) 6:111).  Finally, if desired
(depending on the method to be used for subunit assembly
into polymers) the hydroxyl at the position
corresponding.to the original 5' can be activated by
dissolving the N-protected subunit in a minimal volume
of dry dimethylformamide and adding 2 equivalents of
bis(p-nitrophenyl)carbonate plus 0.2 equivalent of
triethylamine, N-methylimidazole, or N,N-dimethylamino
pyridine.  After 3 h at rt, the reactions mixture is
reduced in volume under vacuum in a warm water bath.
The thick syrup is resuspended in a small volume of
dichloromethane and layered on. a silica gel column,
which is subsequently eluted with a mixture of
dichloromethane/ether (1:1 by vol) 0.2% in
N,N-dimethylaniline by volume.  With this solvent
system, the activated product moves substantially slower
than p-nitrophenol and bis(p-nitrophenyl)carbonate but
much faster than the unreacted starting material.  The
activated product is readily visualized on silica gel
TLC plates simply by exposing to ammonia fumes,
whereupon a yellow nitrophenolate ion is generated
within one to two minutes.

Other ribonucleosides (bases protected where
necessary as in Example 2) are converted to their
corresponding morpholino derivatives by essentially the
same methods -- though varying amounts of methanol must
be added to effect dissolution of the protected

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ribonucleosides before the initial periodate oxidation step.

When the morpholino-type subunits are to be coupled via thiocarbamate linkages, the preferred base-protective groups for the common starting ribonucleosides are: the phenylsulfonylethoxycarbonyl moiety for cytidine and adenosine and the 9-fluorenyl methoxycarbonyl moiety for guanosine.

### Example 6

Preparation of 5'-acetic acid-2',5'-dideoxyribonucleoside subunits.

Triethylamine, pyridine/sulfur trioxide complex, p-nitrophenol, dicyclohexylcarbodiimide, and 40% aqueous dimethylamine are obtained from Aldrich.

3'-O-tert-Butyldimethylsilyl-N-2-(phenylsulfonyl) ethoxycarbonyladenosine (1 mmol), prepared as in Example 12.1, is dissolved in methylene chloride and dimethylsulfoxide (10 mL, 1/1, v/v) at 0°C and triethylamine (3 mmol) and the pyridine/sulfur trioxide complex (3 mmol) is added. The mixture is stirred at this temperature for 1 h, then washed with water, dried over sodium sulfate, and evaporated under reduced pressure. The residue is dissolved in dry pyridine (10 mL) and treated with benzyloxycarbonylmethylenetriphenlyphosphorane (1.5 mmol) for 24 h at 37°C. The mixture is then evaporated to dryness under reduced pressure and the residue dissolved in methylene chloride, washed with dilute HCl and water, then the organic phases are dried over sodium sulfate and solvent removed under reduced pressure. The residue is chromatographed on silica using a gradient of methanol in chloroform (0-20%).

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       The unsaturated ester (1 mmol) from the
previous paragraph is dissolved in ethyl acetate/ethanol
(40 mL, 1/1, v/v) containing cyclohexadiene (5 mL) and
10% palladium on charcoal (100 mg) and the suspension is
5.  vigorously agitated under a nitrogen atmosphere for 5-24
h. The mixture is filtered and the solvents removed
under reduced pressure. The residue is chromatographed
on silica gel using a gradient of methanol in chloroform
(5-50%).

10       To a 0.2-0.5 M solution of the acid from the
previous paragraph in ethyl acetate (or methylene
chloride) p-nitrophenol is added in about 20% excess.
The calculated amount of dicyclohexylcarbodiimide is
added to the solution at 0°C. After 0.5 h the mixture
15  is allowed to come to room temperature and was held
there for 1 h. The dicyclohexylurea which separated is
filtered and washed with solvent. The combined filtrate
and washings are evaporated to dryness under reduced
pressure. The ester may be used directly for coupling
20  reactions or may be purified by short column
chromatography on silica using an isopropanol in
chloroform gradient (0-50%).

       The analogous series of reactions may be
carried out on the other properly protected
25  2'-deoxynucleosides.

       The active ester (1 mmol) is treated with 40%
aqueous dimethylamine (2 mmol) in methanol to form the
N,N-dimethylamide of the N-protected 3'-O-tert-butyl
dimethylsilyl-2',5'-dideoxyadenosine-5'-acetic acid.
30  The solvents are removed by evaporation under reduced
pressure and the residue is desilylated by using the 2M
HF/1M tert-butylammonium fluoride (TBAF) reagent
described in B.L. Gaffney and R.A. Jones, _Tetrahedron_
_Lett_ (1982) __23__:2257. To the 2M HF/1M tert-butylammonium

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fluoride in pyridine (1 mmol of TBAF) is added the
deprotected nucleoside from the previous paragraph.
After stirring for 24 h the reaction is partitioned
between methylene and aqueous sodium bicarbonate.  The
5  organic layer is washed with water, dried over sodium
sulfate, and evaporated to dryness.  The residue is
purified by chromatography on silica using a gradient of
methanol in methylene chloride (5-25%).

## Example 7
10  ## Preparation of Purine and Pyrimidine Pyrrolidones

Potassium t-butoxide, anisoyl chloride,
6-chloropurine, 10% palladium on charcoal, phthaloyl
15  chloride, 2-amino-6-chloropurine, 20% aqueous
tetraethylammonium hydroxide, 25% aqueous
trimethylamine, 2-pyrimidinone, N-bromosuccinimide,
trifluoroacetic acid, 4-nitrophenol, and
N,N-disuccinimydyl carbonate are obtained from Aldrich.
20  Lithium azide is obtained from Eastman Kodak, Rochester,
New York.  C18 reverse phase silica gel is obtained from
Whatman, Hillsboro, Oregon.

7.1 Preparation of (S)-5-[(4-amino-2-oxopyrimidinyl)-
25  methyl]-pyrrolidone (henceforth referred to as the
cytosine pyrrolidone)
Cytosine (2.2 mmol) is dissolved in dimethyl
sulfoxide (2 mL) which contained potassium tert-butoxide
(2 mmol).  This solution is added to a flask which
30  contained (S)-5-(tosyloxymethyl)-2-pyrrolidone (1 mmol,
prepared by the procedure of E. Hardegger and H. Ott,
Helv Chim Acta (1955) 38:312).  After dissolution, the
mixture is stirred at 25°C for 6 h.  The mixture is
neutralized with acetic acid (2 mmol) and evaporated

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under reduced pressure. The residue is taken up in dimethyl formamide (5 mL) and evaporated under reduced pressure, and this procedure repeated three times.

5  7.2 Preparation of N-4 anisoylcytosine pyrrolidone

The mixture from the previous paragraph is dissolved in pyridine or N-methylimidazole (10 mL) and treated with anisoyl chloride (2.8 mmol) at room temperature. After stirring for 2 h, the mixture is

10  quenched with ice (0.5 g). After 5 min, 1 mL of 29% ammonia was added. After 15 min at rt the solution is dissolved in ethyl acetate (15 mL) and washed twice with brine (15 mL). The washings are combined and washed with ethyl acetate (2 x 30 mL), the combined organic

15  layers dried with sodium sulfate and evaporated under reduced pressure. The residue is coevaporated several times with toluene and the residue chromatographed on silica gel with a gradient of methanol in methylene chloride (0-20%).

20

7.3 Preparation of 6-chloropurine pyrrolidone

This compound is prepared as for the alkylation of cytosine except that the alkylation is performed on 6-chloropurine and the mixture was heated at 35-95°C for

25  several h. The mixture is cooled to room temperature, neutralized with acetic acid (2 mmol) and evaporated under reduced pressure, shaken with a mixture of 20% methanol/chloroform and 10% sodium bicarbonate. The aqueous layer is washed with chloroform, the combined

30  organic layers aree dried over sodium sulfate and evaporated under reduced pressure. The residue is chromatographed on silica gel using a gradient of methanol in chloroform (0-25%).

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### 7.4 Preparation of 6-azidopurine pyrrolidone

The 6-chloropurine pyrrolidone (1 mmol) is treated with lithium azide (2 mmol) in dimethyl sulfoxide (2 mL) at 30-100°C for several h. At the end
5 of this time the mixture is evaporated under reduced pressure, dissolved in chloroform, washed with 10% sodium bicarbonate, and the organic layer dried over sodium sulfate and concentrated under reduced pressure. The residue is chromatographed on silica using a
10 gradient of methanol in chloroform (0-25%).

### 7.5 Preparation of adenosine pyrrolidone

The azide from the previous example is hydrogenated by shaking the azide (1 mmol) with 30
15 pounds of hydrogen pressure in a solution of ethanol (10 mL) containing palladium on charcoal (100 mg, 10% by weight Pd) for 24 h. The solution is filtered through celite and the solvent evaporated. The residue is used directly for the next step.

20
### 7.6 Preparation of N-6 phthaloyladenosine pyrrolidone

The phthaloyl group is introduced at the N-6 position by the acylation of the adenosine pyrrolidone (1 mmol) with phthaloyl chloride (1.4 mmol) using the
25 procedure above for the anisoylation of cytidine except that no ammonia was added in the workup.

### 7.7 Preparation of 2-amino-6-chloropurine pyrrolidone

The pyrrolidone tosylate is alkylated with
30 2-amino-6-chloropurine as per the preparation of the 6-chloropurine derivative.

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### 7.8 Preparation of guanine pyrrolidone

The chloropurine pyrrolidone (1 mmol) from the previous paragraph is dissolved in diglyme (10 mL) and 25% aqueous trimethylamine (2 mL). The solution is

5    stirred at rt for 5 h, water (10 mL) is added, and the mixture concentrated to 10 mL under reduced pressure. Acetic acid (2 mL) is added, and the mixture was evaporated under reduced pressure to an oil. The residue which contains tetraethylammonium acetate is

10   used directly for the next step.

### 7.9 Preparation of N-2 acetylguanine pyrrolidone

Guanine pyrrolidone (1 mmol) from the previous paragraph is reacted with acetic anhydride (5 mL) at 

15   reflux for 2 h. The solvent is evaporated and the residue coevaporated with dimethylformamide. The residue is dissolved in methanol (5 mL) saturated with ammonia at 0°C, and the solution is stirred for 2 h at this temperature. The solvent is evaporated under

20   reduced pressure and the residue purified by chromatography on a short column of silica using a gradient of methanol in chloroform (5-50%).

### 7.10 Preparation of 2-amino-6-azidopurine pyrrolidone

25   This compound is prepared from the 2-amino-6-chloropurine pyrrolidone by use of the procedure of Example 7.4, except that the reaction requires longer times and was performed at a higher temperature.

30
### 7.11 Preparation of 2,6-diaminopurine pyrrolidone

This compound is prepared as for the adenosine pyrrolidone by reduction of the 2-amino-6-azido derivative.

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### 7.12 Preparation of N-2 acetyl N-6 phthaloyl 2,6-diamino purine pyrrolidone

The acetyl group is introduced by the method above for N-2 acetylguanine pyrrolidone except that the
5    methanolic ammonia treatment is carried out for 8 h. The solvents are evaporated and the residue is phthaloylated as for the adenosine derivative.

### 7.13 Preparation of inosine pyrrolidone

6-Chloropurine pyrrolidone (1 mmol) was
10   converted to the inosine derivative by the method used for the preparation of the guanine pyrrolidone derivative. The compound is purified by chromatography on silica using a gradient of methanol in chloroform
15   (5-25%).

### 7.14 Preparation of 2-hydroxypyrimidine pyrrolidone

This compound is obtained by the alkylation of 2-pyrimidinone by the procedure used for the preparation
20   of the 6-chloropurine derivative.

### Example 8
### Preparation of Purine and Pyrimidine T-BOC Amino Acids

25   The general procedure described below for the cytosine pyrrolidone may be applied to all properly protected pyrrolidone derivatives. The product, referred to as the t-BOC acid is formed by cleavage of the pyrrolidone ring to an acid and a t-BOC amine.

30
### 8.1 Preparation of the cytosine t-BOC acid

N-4-Anisoylcytosine pyrrolidone (1 mmol) is dissolved in methylene chloride (0.5 M solution) containing di-tert-butyl dicarbonate (2 mmol),

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triethylamine (1 mmol), and dimethylaminopyridine (1
mmol).  The solution is stirred for 10 h at rt.  The
volatiles are removed and the residue purified on silica
using a gradient of methanol in chloroform (0-20%).  The
5   residue is dissolved in tetrahydrofuran (0.2 M solution)
to which is then added lithium hydroxide ( 3 mmol, as a
1 M solution).  After 5 h at rt, the solution is
neutralized by the addition of 10% acetic acid (3 mmol)
and the solvents removed under reduced pressure.  The
10  residue was dissolved in 9N ammonia and stirred at room
temperature for 1-10 h.  The solvent is removed under
reduced pressure and the residue purified by
chromatography on C18 reverse phase silica gel using
water and methanol (or trifluoroethanol) mixtures.

15

8.2 Preparation of uracil t-BOC acid
        This is prepared from the cytosine t-BOC acid
by the following procedure:
        The cytosine t-BOC acid (1 mmol) is dissolved
20  in aqueous acetic acid and treated with sodium nitrite
(1 mmol) at 4°C.  The mixture is stirred for 1 h and
evaporated to dryness under reduced pressure.  The
product is purified by chromatography in C18 reverse
phase silica gel using water and methanol (or
25  trifluoroethanol) mixtures.


8.3 Preparation of 5-bromouracil t-BOC acid
        This compound is obtained from the uracil t-BOC
acid by the following procedure:
30      The uracil t-BOC acid (1 mmol) is dissolved in
DMF (3 mL) and treated with N-bromosuccinimide (1.2
mmol).  The solution is allowed to stand at room
temperature for 16 h.  After removal of the solvents
under reduced pressure the product is purified by

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chromatography on C18 reverse phase silica gel using
water and methanol (or trifluoroethanol) mixtures.

8.4 Preparation of N-protected t-BOC acid

5       This general procedure for acylating the
exocyclic amino groups of the recognition moiety is also
applicable to the protection of the adenosine derivative.
        The cytosine t-BOC acid (1 mmol) from the
previous paragraph is dissolved in N-methylimidazole or
10  pyridine (5 mL) and treated with chlorotrimethylsilane
(5 mmol).  After 15 min at room temperature the solution
is treated with 2-(4-nitrophenyl)ethylchloroformate (3
mmol, obtained from F. Himmelsbach and W. Pfleiderer,
Tetrahedron Lett (1983) 24:3583).  The reaction iss
15  maintained at room temperature for 8 h, then cooled in
an ice bath, and water (1 mL) was added.  After 5 min, 1
mL of concentrated ammonia is added and the mixture
stirred at rt for 15 min.  The reaction is then
evaporated to dryness and the residue dissolved in
20  water.  The solution is made acidic with 2 M HCl and
extracted with chloroform.  The combined organic
extracts are dried over sodium sulfate and evaporated
under reduced pressure.  The residue is purified by
chromatography on silica using a gradient of methanol in
25  chloroform (5-50%).
        For protection of the guanine and
2,6-diaminopurine derivatives, the above procedure was
modified so that the reaction was carried out in
pyridine and the acylating agent was 9-fluorenylmethyl
30  chloroformate.

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## Example 9
### Preparation of Purine and Pyrimidine Amino Acids

The following procedure is applicable to all
the derivatives.  It removes the t-BOC group and frees
the primary amino group of the backbone.

The t-BOC acid (1 mmol) was dissolved in 20%
trifluoroacetic acid in methylene chloride (5 mL) and
stirred at rt for 30 min.  Toluene (3 mL) is added and
the solvents are evaporated under reduced pressure to
give the amino acid trifluoroacetate salt.  This is used
directly for coupling reactions.


## Example 10
### Preparation of Subunits for Assembly
### of Achiral Acyclic Polynucleotide Analogs

#### 10.1    Preparation of the Cytidine Analog

Lithium borohydride, di-tert-butyl dicarbonate
and bis(triphenylphosphine) palladium II chloride were
obtained from Aldrich Chemical Co., Milwaukee, WI.
2-Amino-5-bromopyrimidine is prepared as per T.
Nishikawa, Chem. Pharm. Bull, 9:38 (1961).

2-amino-5-bromopyrimidine is benzoylated at the
exocyclic amino group by the general method in Example 2
using benzoyl chloride.  The product benzamide (25
mmole) is added to a 45 mL pressure vessel containing
triethylamine (10 mL), benzene (10 mL), and
bis(triphenylphosphine) palladium II chloride (0.375
mmole).  The bomb is flushed with argon, sealed, and
pressurized to 600 psi with carbon monoxide and then
pressurized to 1200 psi with hydrogen.  The reaction
vessel is heated in an oil bath with stirring at 145°C.

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after all gas absorption had ceased, the reaction is cooled, and the gases vented slowly. After addition of anhydrous ether, the reaction mixture is filtered and evaporated in vacuo. The product was isolated following chromatography in silica gel using a gradient of isopropanol in methylene chloride (0-20%).

The 2-benzamidopyrimidine-5-carboxaldehyde (1 mmol) from the previous paragraph is dissolved in ethanol (5 mL) and treated with 4-aminobutyric acid (1 mmole) and triethylamine (1 mmol). After stirring for one hour, the mixture is hydrogenated at 30 psi using 10% palladium on carbon (100 mg). After the absorption of hydrogen has ceased, the reaction is filtered and the solvents removed under reduced pressure. The residue is dissolved in methanol which is saturated with ammonia at 0°C and stirred at room temperature for 10 h. After evapoaration of the solvents, the residue iss purified by chromatography on C18 silica gel using methanol water mixtures buffered to pH 7 with ammonium acetate (0.2 M).

The amino acid (1 mmol) from the previous paragraph is dissolved in ethanol (3 mL) containing di-tert-butyl dicarbonate (1 mmol). Triethylamine (1.5 mmol) is added slowly at room temperature. After the evolution of carbon dioxide has ceased, the solvents are removed in vacuo and the residue used directly for the next reaction.

The t-BOC-amino acid from the previous paragraph is acylated on the exocyclic amino group by the general procedure in Example 2 using 2-(phenylsulfonyl)-ethoxycarbonyl chloride. The product may be purified by chromatography on silica gel using a gradient of methanol in chloroform (0-50%).

The 2-(phenylsulfonyl)-ethoxycarbonyl-protected BOC amino acid from the previous paragraph was converted

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into the p-nitrophenyl or N-hydroxysuccinimidoyl active
ester by the procedures in Examples 11.3 and 11.4.

10.2.   Preparation of the Uridine Analog

5          Ethyl 6-hydroxynicotinate is prepared as per H.
Gault, J. Gilbert, and D. Briaucourt, C.R. Acad. Sci.,
Paris, Ser. C, 266:131 (1968).   Lithium borohydride is
obtained from Aldrich.   Manganese dioxide is prepared as
per Vereshchagin et al, Zhurnal Arganicheskoi Khimil,
10   8:1129 (1972).   Dowex resin is obtained from Bio-Rad
Laboratories, Richmond, CA.

          Ethyl 6-hydroxynicotinate (49 mmole) in
tetrahydrofuran (10 mL) is added dropwise to a
suspension of lithium borohydride (100 mmol) in
15   tetrahydrofuran (THF) (75mLO at room temperature under
nitrogen.   The viscous mixture is stirred at 25°C for 16
hours.   Then, 60 mL of 20% acetic acid is added to the
cooled suspension.   The THF is evaporated under reduced
pressure and the remaining aqueous solution is applied
20   to an ion exchange column (Dowex-50W-X8) to remove
lithium ion.   The column is washed carefully with 250 mL
of water, which is then evaporated.   Boric acid is
removed from the resulting residue by repeated
evaporation with methanol.   The product is purified by
25   distillation under reduced pressure.

          The benzylic alcohol (66 mmol) prepared in the
previous paragraph is dissolved in ether (250 mL) and
treated slowly (exothermic) with a slurry of manganese
dioxide (54 g) in ether (100 mL).   After twelve hours at
30   room temperature, the mixture is filtered, the solvent
removed under reduced pressure, and the residue
distilled in vacuo to give 6-hydroxynicotinaldehyde.

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The aldehyde from the previous paragraph is
reacted with 4-amino butyric acid as for the preparation
of the cytidine analog in Example 11.1

   The amino acid from the previous paragraph was
5  converted to the corresponding BOC-derivative as for the
preparation of the cytidine analog in Example 10.1

   The BOC-amino acid from the previous paragraph
iss converted into the p-nitrophenyl or
N-hydroxysuccinimidoyl active ester by the procedures in
10 Example 11.3 and 11.4.


## Example 11

### Preparation of 5'-protected, 3'-activated 2'-deoxynucleo- sides.

15

### 11.1  2'-deoxynucleoside 5'-O-dimethoxytrityl derivatives

   The procedure below is general for the
preparation of 2'-deoxynucleoside 5'-O-dimethoxytrityl
20 derivatives:

   N-2 9-Fluorenylmethoxycarbonyl-2'-
deoxyguanosine (1 mmol) is dissolved in anhydrous
pyridine (2.0 mL) and kept at 0°C.  Dimethoxytrityl
chloride (1.2 mmol) is added in portions (0.15 mmol)
25 during an 8 hour period.  After an additional 2 hours
methanol (0.5 mL) was added and the solution is
concentrated at reduced pressure.  The residue is
treated with a mixture of sodium bicarbonate (2 mL, 5%)
and methylene chloride (5 mL).

30   The aqueous layer is extracted with methylene
chloride (2x5 mL) and the combined organic layers are
dried over sodium sulfate and evaporated.  The residue
is coevaporated several times with toluene and the
product is purified by chromatography on silica gel

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using a gradient of methanol in methylene chloride
(0-10%).

## 11.2 5'-O-dimethoxytrityl-2'-deoxynucleoside 3'-O-(4-nitrophenyl)carbonates.

The preparation of 5'-O-dimethoxytrityl-2
-deoxycytidine 3'-O-(4-nitrophenyl)carbonates is
described in the following procedure which is general
for the preparation of the 4-nitrophenylcarbonates.  Bis
(4-nitrophenyl)carbonate and N,N-dimethylaminopyridine
are obtained from Sigma.

5'O-Dimethoxytrityl-N-2-(4-nitrophenyl)-
ethoxycarbonyl-2'-deoxycytidine (1 mmol) is dissolved in
anhydrous dimethylformamide (5 mL) at room temperature
and treated with bis(4-nitrophenyl)carbonate (2 mmol)
and then evaporated under reduced pressure.  The residue
is dissolved in dimethylformamide (5 mL) and
N,N-dimethylaminopyridine (0.1 mmol) is added.  The
yellow solution is evaporated and allowed to react for 1
h.  The reaction mixture is dissolved in chloroform (10
mL) and washed with aqueous HCl (5 mL, 0.1 M).  The
organic layer is washed twice with aqueous NaOH (5 mL,
0.1 M), water (2 mL), dried over sodium sulfate and
evaporated.  The residue is applied to a silica gel
column and eluted with a gradient of isopropanol in
chloroform (0-10%).  The fractions containing the
product are pooled, evaporated, dissolved in chloroform
(10 mL) and the aqueous NaOH washes are repeated to
remove traces of 4-nitrophenol.  The organic layer is
washed with water, dried over sodium sulfate and
evaporated.  The product is homogeneous by TLC and is
used directly for preparation of the dimeric
carbonates.

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For the preparations of the carbonates of
nucleosides containing the FMOC group it is sometimes
advantageous to use N-methylimidazole as the catalyst
rather than N,N-dimethylaminopyridine.

5

### 11.3 N-protected t-BOC p-nitrophenyl ester

The following two procedures prepare active
esters for coupling of subunits.  It is general for all
10    the t-BOC acid derivatives.

To a 0.2-0.5 M solution of the t-BOC acid from
Example 8.4 in ethyl acetate (or methylene chloride),
p-nitrophenol is added in about 20% excess.  The
calculated amount of dicyclohexylcarbodiimide is added
15    to the solution at 0°C.  After 0.5 h the mixture is
allowed to come to rt and was held there for 1 h.  The
dicyclohexylurea which separated is filtered and washed
with solvent.  The combined filtrate and washings are
evaporated to dryness under reduced pressure.  The ester
20    may be used directly for coupling reactions or may be
purified by short column chromatography on silica using
an isopropanol-in-chloroform gradient (0-50%).

### 11.4 N-protected t-BOC N-hydroxysuccinimidoyl ester

25    To a solution of the t-BOC acid from Example
8.4 (1 mmol) in acetonitrile (5 mL) is added pyridine (1
mmol) and N,N'-disuccinimidyl carbonate (1 mmol).  The
solution ia stirred at rt for 3 h.  The solvents are
removed under reduced pressure and the residue dissolved
30    in chloroform, the organic phase washed with 0.1 M HCl
and water, dried over sodium sulfate, and evaporated to
dryness.  The esters are sufficiently pure to be used
directly.

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## Example 12
## Formation of a carbonate intersubunit linkage.

The preparation of cytidinyl-(3'-5')-cytidine
carbonate is described in the procedures below.  This is

5  a general procedure for the formation of the carbonate
intersubunit linkage and the deprotection of the
oligocarbonate.  Note that the base protecting groups
must be 2-(4-nitrophenyl)ethoxycarbonyl, 2-(phenyl
sulfonyl)ethoxycarbonyl, or FMOC.  Tert-butyl

10  dimethylsilyl chloride is obtained from Aldrich.


### 12.1 Preparation of 2'-deoxynucleoside 3'-O-tert-butyldi-methylsilyl derivatives.

The preparation of 3'-O-tert-butyldimethylsilyl-

15  N-2-(4-nitrophenyl)ethoxycarbonyl-2'-deoxycytidine is
described in the following procedure which is a general
procedure for the preparation of 2'-deoxynucleoside-3'-
O-tert-butyldimethylsilyl derivatives:

To a stirred solution of 5'-O-dimethoxytrityl-

20  N-4-(4-nitrophenyl)-ethoxycarbonyl-2'-deoxycytidine
(1 mmol) and imidazole (3.5 mmol) in dry
dimethylformamide or tetrahydrofuran (10 mL) is added
dropwise a solution of tert-butyldimethylsilyl chloride
(3 mmol) in dry DMF (10 mL).  The reaction is stirred

25  for 1 h, quenched with ice, extracted with methylene
chloride, washed with water and dried over sodium
sulfate.  The organic layer is evaporated to dryness
under reduced pressure and purified by chromatography
over silica gel with methylene chloride as eluant.

30  To a solution of the 5'-O-dimethoxytrityl
derivative from the previous paragraph (1 mmol) in
methylene chloride (5 mmol) is added a solution of zinc
bromide in methylene chloride (10 mL, 1 M) containing
methanol (15 % v/v).  The reaction mixture is stirred

for 30 min, quenched with ice, and extracted with
methylene chloride. The organic layer is washed with
water, dried over sodium sulfate, and evaporated to
dryness under reduced pressure. The product is purified
5    by chromatography over silica gel using a gradient of
methanol in methylene chloride (0-10%).

5'-O-dimethoxytrityl-N-2-(4-nitrophenyl)ethoxy
carbonyl-2'-deoxycytidine-3'-O-(4-nitrophenyl)carbonate
(1.5 mmol) and 3'-O-tert-butyldimethylsilyl-N-4-(4-
10   nitrophenyl)ethoxycarbonyl-2'-deoxycytidine (1 mmol) are
dissolved in dimethylformamide (5 mL) and the solvent
removed under reduced pressure. This is repeated two
times. The residue is redissolved in dimethylformamide
(5 mL) and treated with N,N-dimethylaminopyridine (0.5
15   mmol) or N-methylimidazole (1 mmol). The solvent is
removed by evaporation under reduced pressure and the
reaction allowed to sit overnight at rt. The residue is
dissolved in chloroform (20 mL) and washed with aqueous
HCl (2 mL, 0.1 M), water (2 mL), aqueous NaOH (2 x 2 mL,
20   0.2 M), water (2 mL), and the organic layer dried over
sodium sulfate and evaporated. The residue is purified
on silica gel using a gradient of isopropanol in
chloroform (0-30%). The isolated product is homogeneous
on TLC and by 400 MHz NMR.

25           The residue is desilylated by using the 2 M
HF/1M tert-butylammonium fluoride (TBAF) reagent
described in B.L. Gaffney and R.A. Jones, Tetrahedron
Lett (1982) 23:2257. To the 2M HF/1M tert-butylammonium
fluoride in pyridine (1 mmol of TBAF) is added the
30   deprotected nucleoside from the previous paragraph.
After stirring for 24 h, the reaction is partitioned
between methylene chloride and aqueous sodium
bicarbonate. The organic layer is washed with water,
dried over sodium sulfate, and evaporated to dryness.

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The residue is purified by chromatography on silica
using a gradient of methanol in methylene chloride
(5-25%).

5       The 3'-hydroxydinucleoside carbonates are
converted to the 3'-(4-nitrophenyl)-carbonates by the
method in Example 11.  These derivatives may be reacted
with the 5'-hydroxyl of a nucleoside or oligonucleoside
carbonate to prepare higher order oligonucleoside
carbonates.

10
                        Example 13
        Formation of a carbamate intersubunit linkage.
            Bis(p-nitrophenyl)carbonate, p-anisoyldiphenyl
methyl chloride, N,N-dimethylaniline (DMA), and
15  triethylamine are available from Aldrich Chemical Co.
            The required
5'-amino-2',5'-dideoxyribonucleoside (employing acetyl
or benzoyl groups for base protection where necessary)
(1 mmol) prepared in Example 3 above, is treated with
20  p-anisoyl diphenylmethyl chloride (1.2 mmol) in
pyridine.  After 12 h the solvent is evaporated and the
residue is redissolved in chloroform.  This solution is
washed once each with aqueous $NaHCO_3$ and water, then
dried over $Na_2SO_4$.  After evaporation of the
25  solvent, the residue is chromatographed on silica gel
eluting with chloroform/methanol/1% triethylamine
mixtures.
            The 5'-tritylated aminonucleoside (1 mmol) is
evaporated twice from DMF, then treated with
30  bis-(p-nitrophenyl)carbonate (2 mmol) in the presence of
triethylamine (or N,N-dimethylaminopyridine, or N-methyl
imidazole)(catalytic amount) using DMF as the solvent.
After 3 h, the solvent is evaporated and the residue
dissolved in chloroform.  This solution is washed twice

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with 0.01 N aqueous NaOH, once with water, then dried over $Na_2SO_4$. The solvent is removed by rotovap and the residue is chromatographed on silica gel eluting first with a chloroform/0.1% DMA mixture, then with a

5   chloroform/methanol/0.1% DMA solvent system. The appropriate fractions are combined and evaporated to dryness. The residue is dissolved in a minimum of THF and this solution is added to an excess of hexanes. The precipitated activated nucleoside is collected by

10  filtration and dried under vacuum.

The required 5'-aminonucleoside (1.1 mmol), as prepared in Example 3 above, is twice evaporated from DMF. The activated nucleoside (1 mmol) is added to the reaction vessel and the solids are dissolved in DMF.

15  The solution is concentrated to a small volume and allowed to stand overnight. The solvent is completely removed under vacuum and the resulting residue is dissolved in chloroform. This solution is twice washed with 0.01 N aqueous sodium hydroxide, once with water,

20  then dried over solid sodium sulfate. The solvent is removed by rotovap, and the residue is chromatographed on silica gel eluting with the appropriate methanol/chloroform/1% triethylamine solvent system. The fractions containing the dimer nucleoside are

25  combined, then evaporated to dryness. The residue is dissolved in a minimum of THF and this solution is added to hexanes. The precipitate is collected and dried under vacuum.

## Example 14

30

## Formation of a thiocarbamate intersubunit linkage.

N,N-dimethylaminopyridine (DMAP) is purchased from Aldrich Chemical Co.

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Formation of the thiocarbamate intersubunit
linkage of the 5'-aminonucleosides prepared in Example 3
is achieved in the same manner as described above in
Example 13 with the following alterations in procedure.
5    The 5'-aminonucleosides employed have as base-protecting
moieties either 2-(phenylsulfonyl)ethoxycarbonyl (for
deoxycytidine and deoxyadenosine) or 9-FMOC (for
guanosine and 2,6-diamino-2'-deoxyriboside) groups. The
preparation of these molecules are described above
10   (Example 3). Activation of the 5'-tritylaminonucleoside
(1 mmol) is achieved with p-nitrophenylchlorothioformate
(as prepared by G Hilgetag and r. Phillippson, Monatsber
Deut Akad Wiss Berlin (1964) 6:897) (1.2 mmol) in the
presence of triethylamine (2 mmol) and the DMAP
15   (catalytic amount) in DMF. The coupling step employs
this activated monomer (1 mmol) with the requisite
5'-aminonucleoside (1.1 mmol) using DMAP or N-methyl
imidazole as catalyst and DMF as the solvent. In this
aqueous workup of these steps, the 0.01 N aqueous NaOH
20   washes are omitted, employing in their place water
washes.


## Example 15
Formation of carbamate and thiocarbamate intersubunit
25   linkages between morpholino type subunits.
N-methylimidazole is purchased from Aldrich
Chemical Co.
The requisite dry, N'-protected, 5'-free
alcohol morpholino type nucleoside (employing acetyl or
30   benzoyl groups for base protection where necessary)
(1 mmol), prepared in Example 5 above is treated with
bis-(p-nitrophenyl)carbonate and triethylamine (or DMAP
or N-methylimidazole) (catalytic amount) in DMF under
anhydrous conditions. The solution is stirred for 3 h,

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then evaporated to dryness.  The residue is dissolved in
chloroform and the solution is washed twice with 0.01 N
aqueous NaOH, once with water, then dried over
$Na_2SO_4$.  The solvent is removed by rotovap and the
5   residue is chromatographed on silica gel eluting with an
appropriate chloroform/isopropanol/0.1% DMA mixture.
The fractions containing the desired activated
nucleoside are combined, evaporated, and the resulting
solid is dissolved in a minimum of THF.  The THF
10  solution is added to an excess of hexanes and the
resulting precipitate is collected and dried.

    The required 5'-free hydroxy, N'-free
morpholino type nucleoside (using the identical base
protecting groups listed above in this example) (1.1
15  mmol), after evaporation twice from DMF, is treated with
the 5'-(p-nitrophenoxycarbonyl)morpholino subunit (1
mmol) in DMF.  The volume of the reaction solution is
reduced under vacuum to a small volume.  If required, a
catalytic amount of N-methylimidazole or triethylamine
20  is added to the reaction vessel.  The resulting solution
is allowed to stand overnight at rt.  The remaining
solvent is evaporated, and the residue is dissolved in
chloroform.  The chloroform solution is washed twice
with 0.01 N aqueous NaOH, once with water, and then
25  dried over $Na_2SO_4$.  The solvent is removed by
rotovap and the residue is chromatographed on silica gel
eluting with chloroform/methanol solvent mixtures.  The
fractions containing the desired dimer are combined,
then rotovaped to dryness.  The residue is dissolved in
30  a minimum of THF and the solution is added to an excess
of hexanes.  The precipitate is collected and dried
under vacuum.

    Morpholino subunits linked by thiocarbamate
moieties are prepared as related above in this example

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with the following experimental alterations.  The base-
protecting groups are either
1-(phenylsulfonyl)ethoxycarbonyl (for deoxycytidine and
deoxyadenosine) or 9-FMOC (for deoxyguanosine and

5   2,6-diamino-2'-deoxyriboside) groups.  The preparation
of these molecules is described above (Example 5).
Activation of the N-protected morpholino nucleoside
(1 mmol) is achieved with p-nitrophenylchlorothioformate
(1.2 mmol) in the presence of triethylamine (2 mmol) and

10  DMAP or N-methyl imidazole (catalytic amount) in DMF.
The coupling step employs this activated monomer (1
mmol) with the requisite N'-free morpholino nucleoside
(1.1 mmol) using DMAP or N-methyl imidazole as catalyst
and DMF as the solvent while warming the solution to ·

15  35-40°C.  In the aqueous workup of these steps, the 0.01
N aqueous NaOH washes are omitted, employing in their
place water washes.


## Example 16

### Preparation of an ester subunit linkage.

20        N,N-dimethylaminopyridine and N-methylimidazole
are obtained from Aldrich.
          The coupling of two subunits is performed by
reaction of the 5'-N,N-acetamide-3'-hydroxy derivative
with the  with the 3'-silylated-5'-active ester.  These

25  are prepared as in Example 6.  The reagents (1 mmol
each) are coevaporated several times with
dimethylformamide, then dissolved in dimethylformamide
(5 mL) and treated with N,N-dimethylaminopyridine (0.2
mmol) or N-methylimidazole (1 mmol).  The solvent is

30  removed under reduced pressure and the reaction allowed
to stand at room temperature for 2 h.  The residue is
dissolved in chloroform and washed with dilute HCl,
water, and the organic phase dried with sodium sulfate

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and solvents evaporated under reduced pressure.  The
residue is purified by chromatography on silica gel
using a gradient of methanol in chloroform.

    The chain may be elongated by desilylation as
5 in Example 6, and treatment of the resulting 3'-free
hydroxy compound with the active ester as in the
previous paragraph.


## Example 17
### Formation of a t-BOC amide-linked dimer

10     A fully protected t-BOC N-hydroxysuccinimidoyl
ester or p-nitrophenyl ester prepared as in Example 11.3
and 11.4 (1 mmol) is dried by several coevaporations
with dry dimethyformamide.  The protected amino acid
trifluoroacetate salt from Example 9 (1 mmol) is treated
15 similarly.  The two components are separately dissolved
in dry dimethylformamide (2 mL), mixed, and treated with
diisopropylethylamine (1.0 mmol).  The solution is
stirred at room temperature for 1 h, then the solvent is
removed by evaporation under reduced pressure, the
20 residue dissolved in chloroform and washed with dilute
HCl.  The organic layer is dried over sodium sulfate,
evaporated to dryness under reduced pressure, and the
residue purified by chromatography on silica gel using a
gradient of methanol in chloroform (5-50%).
25 Alternatively the t-BOC dimer acid may be purified by
chromatography on C18 reverse phase silica gel using
water and methanol (trifluoroethanol) mixtures.


30 17.1 Preparation of the t-BOC dimer active ester
    The acid from the previous paragraph is
converted into the N-hydroxysuccinimidoyl ester or
p-nitrophenyl ester by application of the general
methods in Example 11.3 and 11.4.

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17.2 <u>Preparation of the t-BOC dimer amino acid trifluoro</u>
<u>acetate</u>

The t-BOC dimer acid is treated with trifluoro
acetate as per the general procedure in Example 9.

17.3 <u>Preparation of a t-BOC tetramer acid</u>

The t-BOC dimer active ester and the dimer acid
trifluoroacetate are coupled to the t-BOC tetramer acid
by the general procedure. Purification is best effected
by chromatography on C18 reverse phase silica gel using
water and methanol (or trifluoroethanol) mixtures
buffered to pH 7.0 with triethylammonium acetate. In
this manner chains of any length may be prepared by
coupling an active ester with a free amine component.

Example 18

<u>Geometric assembly of an 8-subunit carbamate-linked</u>
<u>polymer</u>

DEAE cellulose is purchased from Sigma Chemical
Co. Preparative TLC plates are a product of EM Science
purchased from VWR Scientific. HPLC equipment, columns,
and supplies are available from Beckman Instruments, Inc.

The requisite carbamate-linked dimer (0.2 mmol)
prepared as described in example 12, is treated with 4
mL of a 1/1/1 mixture of methanol/THF/glacial acetic
acid at rt for 12 h. The solvent is removed under
vacuum and the residue is taken up in a minimum of THF.
The THF solution is added to a large volume of hexanes
and the resulting precipitate is collected and dried.
The acetate salt is dissolved in a 4/1 THF/ethanol
mixture and DEAE cellulose (0.8 mmol of the base) is
added to the vessel. After stirring for 20 min, the
heterogeneous mixture is filtered and the filtrate
evaporated to dryness. The residue is dissolved in a

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minimum of 4/1 THF/ethanol and this solution is added to
hexanes. The solid is collected and dried. The
precipitation procedure is repeated once.

5    The required N-5'-trityl dimer (0.1 mmol) (as
prepared in Example 12) is evaporated twice from DMF,
then treated with bis(p-nitrophenyl)carbonate (2 mmol)
in the presence of triethylamine (or DMAP or N-methyl
imidazole) (catalytic amount) using DMF as the solvent.
after 3 h the solvent is evaporated and the residue
10   dissolved in chloroform. This solution is washed twice
with 0.01 N aqueous NaOH, once with water, then dried
over $Na_2SO_4$. The solvent is removed by rotovap and
the residue is chromatographed on silica gel eluting
with a chloroform/isopropanol or methanol/0.1% DMA
15   mixture. The appropriate fractions are combined and
evaporated to dryness. The residue is dissolved in a
minimum of THF and this solution is added to an excess
of hexanes. The precipitated activated dimer is
collected by filtration and dried under vacuum.

20       The 5'-amino dimer nucleoside prepared above
(0.11 mmol) is twice evaporated from DMF. The activated
dimer (0.1 mmol) is added to the reaction vessel and the
solids are dissolved in DMF. The solution is
concentrated to a small volume and allowed to stand
25   overnight. The solvent is completely removed under
vacuum and the resulting residue is dissolved in
chloroform. This solution is twice washed with 0.01 N
aqueous sodium hydroxide, once with water, then dried
over sodium sulfate. The solvent is removed by rotovap
30   and the residue is chromatographed on a preparative TLC
plate eluting with the appropriate
methanol/chloroform/1% triethylamine solvent system.
The band containing the tetramer nucleoside is eluted
and the solvent is evaporated. The residue is dissolved

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in a minimum of THF and this solution is added to
hexanes.  The precipitate is collected and dried under
vacuum.

The following transformations are performed
5  using the procedures enumerated above in this example.
The requisite N-5'-trityl tetramer nucleoside (0.06
mmol) is treated with 1/1/1 THF/methanol/glacial acetic
acid, then purified to afford the 5'-aminotetramer
nucleoside.  Another aliquot of the N-5'-trityl tetramer
10 nucleoside (0.05 mmol) is activated with bis(p-nitro
phenyl)carbonate and subsequently purified.  The two
tetramers are coupled, worked up, and further
purification is achieved either by chromatography on a
preparative TLC plate or by HPLC chromatography.  the
15 chromatographed material is isolated as a solid,
dissolved in a minimum of THF, and then precipitated
from hexanes.  The octomer nucleoside is collected and
dried.

The octamer is configured for further use as
20 follows.  The N-5'-trityl octomer nucleoside is treated
with 1/1/1 THF/methanol/glacial acetic acid and purified
employing the conditions listed above.  The 5'-amino
octomer nucleoside (0.01 mmol) is treated with succinic
anhydride (0.02 mmol) in pyridine at room temperature
25 for two hours.  The solvent is removed by rotovap and
the residue is evaporated several times from ethanol.
The residue is taken up in 3/1 THF/ethanol and added to
a 2/1 hexane/benzene mixture.  The solid is collected
and dried.  The N-5'-succinylated octamer nucleoside
30 (0.01 mmol) is treated with a 1/1 mixture of
pyridine/concentrated ammonia (1 mL).  After standing
overnight the solvent is removed by rotovap and the
residue is evaporated from ethanol several times.  The
crude solid is purified using reverse phase (RP-18) HPLC

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chromatography.   The elutants are evaporated and the
residue is taken up in DMSO and precipitated from a 1/1
hexane/benzene solvent mixture.   The solid is collected
and dried.

5

## Example 19
### Preparation of a support-bound cleavable linker suitable for stepwise and stepwise/block assembly of polymers: activation of support and attachment of linker.

10

Long-chain alkylamine-derivatized controlled
pore glass (Cat. No. 24875) is obtained from Pierce
Chemical Co.  2,2-sulfonyldiethanol and
dicyclohexylcarbodiimide are obtained from Aldrich
15  Chemical Co.

The amino groups of the glass support
(approximately 40 mmol per gram of support) are reacted
with succinic anhydride essentially by the method of
Matteucci and Caruthers (J Amer Chem Soc (1981)
20  103:3185) to give free carboxylic acid termini.
One-tenth mol of 2,2'-sulfonyldiethanol (60 % by weight
in water) is dried by mixing with three volumes of
dimethylformamide (DMF), reducing to a thick syrup under
vacuum in a warm water bath, adding a second quantity of
25  DMF, reducing to a thick syrup, and repeating the
process a third time.  Dry DMF is then added to give a
final sulfonyldiethanol concentration of 2 M.  A mixture
of 1 ml of the sulfonyldiethanol solution plus  0.2 g of
dicyclohexylcarbodiimide is added to 1 g of the dry
30  controlled pore glass support carrying carboxylic acid
residues and the slurry mixed by rocking or tumbling
(not stirring) overnight at rt.  Thereafter, the glass
support is thoroughly washed with methanol and dried.

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## Example 20

### Preparation of a 14-subunit carbonate-linked polymer targeted against a conserved sequencein the AIDS viral genome: stepwise assembly on a solid support: addition of the first subunit and chain extension.

5

N-methylimidazole,
4-(N,N-dimethylamino)pyridine (DMAP), and
1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) are obtained
10  from Aldrich Chemical co.  Methyl p-nitrophenyl
carbonate is prepared from methyl chloroformate and
p-nitrophenol.

The 2'-deoxycytidine subunit(0.1 mmol)
prepared as in Example 4.2 (wherein the N4 carries a
15  p-nitrophenethoxycarbonyl moiety, the 5' oxygen carries
a di(p-methoxy)trityl moiety (DMT), and the 3' oxygen
carries a p-nitrophenoxycarbonyl moiety) is dissolved in
a minimal volume of dry tetrahydrofuran (THF) or
dimethylformamide and added to 0.5 g of thoroughly dried
20  controlled pore glass support carrying a cleavable
linker, prepared as in Example 19.  Catalyst (either 1
mmol of N-methylimidazole or 0.1 mmol of DMAP) is
introduced and the slurry is mixed by rocking or
trembling (not stirring) for 2 hours.  The slurry is
25  next filtered, and the solid washed thoroughly with THF.

Any unreacted hydroxyls are capped by adding
two ml of THF 1 M in methyl p-nitrophenyl carbonate and
0.5 M in DMAP and mixing for 20 min at rt.  Thereafter,
the glass support is washed with THF and filtered.

30  The dimethoxytrityl at the 5' terminus is then
removed by washing the support with dichloromethane
followed by treatment with 5 ml of 0.2 M dichloroacetic
acid in dichloromethane for 5 min at rt.  The glass
support is next washed with dichloromethane and filtered.

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Subsequent subunits, prepared as in Example 1 and activated as in Example 2, are added in a like manner and in the following order: G,A,T,A,A,C,A,T,T, T,T,T,C, (G protected with the FMOC moiety, A and C
5    protected with the p-nitrophenethoxy carbonylmoiety).

### Example 21
Preparation of a 19-subunit carbamate-linked polymer
targeted against a conserved sequence in the AIDS viral
10   genome.  Stepwise assembly of oligomer blocks on a solid
support:  addition of the first subunit and chain
extension.

The  5'-amino-2',5'-dideoxycytidine subunit
(0.2 mmol), prepared as in Example 3 (wherein the N4
15   carries a benzoyl moiety, the 5'-amine carries a
p-methoxytrityl moiety, and the 3' oxygen carries a
p-nitrophenoxycarbonyl moiety) is dissolved in a minimal
volume of dry THF and added to 0.5 g of dried controlled
pore glass support carrying a cleavable linker, prepared
20   as in Example 19.  Catalyst (either 1 mmol
N-methylimidazole or 0.1 mmol of DMAP) is introduced and
the slurry mixed by rocking or tumbling for 2 h.  The
slurry is next filtered, and the solid washed thoroughly
with THF.

25       The mono-p-methoxy trityl is removed by washing
with dichloromethane, followed by treatment with 5 ml of
0.2 M dichloroacetic acid in dichloromethane at rt for 1
min.  The support is then washed with dichloromethane
and filtered.  The 5' amino termini are converted to
30   their free amine form by a brief wash with 3 ml of THF
containing 1% by volume of diisopropylethylamine,
followed by washing with THF and filtration.

0.1 mmol of 3' p-nitrophenoxycarbonyl-activated
dimer having the sequence 5'-A-G-3', prepared as in

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Example 13 (wherein the guanine N2 is protected with an acetyl moiety and the adenine N6 is protected with a benzoyl or a p-nitrobenzoyl moiety), is dissolved in a minimal volume of dry THF and added to the glass support
5   and mixing is carried out for 2 h (no catalyst is added for this and subsequent coupling steps). The slurry is next washed with THF and filtered.

        Any unreacted amine moieties are capped by adding 2 ml of THF 2 M in p-nitrophenyl acetate and
10  mixing at rt for 20 min. Thereafter, the glass support is washed with THF and filtered.

        The mono-methoxytrityl at the 5' terminus is removed with dichloroacetic acid, as before.

        Subsequent activated dimeric subunits, prepared
15  as in Example 17 (wherein the exocyclic ring nitrogen of cytosine is protected with a benzoyl moiety and the exocyclic ring nitrogen of A is protected with a benzoyl or a p-nitrobenzoyl moiety), and added in a like manner and in the following order:  A-T, C-A, T-A, T-T, T-T,
20  A-C, A-C, C-A (5' to 3').

        Subunits having base exocyclic ring nitrogen protective groups removable by strong nonnucleophilic bases (e.g., p-nitrophenethoxycarbonyl or phenylsulfonyl ethoxycarbonyl for A and C, and FMOC for G) can also be
25  used for assembling polymers by the procedure above. However, polymers having these alternative protective groups are generally deprotected by treatment with DBU rather than by ammonium hydroxide.


30

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## Example 22

Preparation of a 19-subunit amide-linked polymer
targeted against a conserved sequence in the AIDS viral
genome.  Stepwise assembly of oligomer blocks on a solid
support:  addition of the first subunit and chain
extension.

A cytosine-containing acyclic-backboned subunit
(0.2 mmol), prepared as in Example 11.3 (wherein the N4
of the cytosine carries a 2(P-nitrophenyl)ethoxycarbonyl
moiety, the amine of the backbone carries a
t-butoxycarbonyl moiety, and the carboxyl is in the form
of a p-nitrophenyl ester) is dissolved in a minimal
volume of dry THF and added to 0.5 g of dried controlled
pore glass support carrying a cleavable linker, prepared
as in Example 19.  Catalyst (either 1 mmol of
N-methylimidazole or 0.1 mmol of DMAP) is introduced and
the support mixed for 2 h, filtered, and the solid is
washed thoroughly with THF.

The t-BOC is removed by washing the support
with dichloromethane followed by treatment at rt with 5
ml of 20% by volume trifluoroacetic acid in methylene
chloride for 30 min.  The support is washed with
dichloromethane and filtered.  The support-bound amino
terminus is converted to the free amine form by a brief
wash with 3 ml of THF containing 1% by volume of
diisopropylethylamine, followed by a THF wash.

The activated dimeric subunit (0.1 mmol) having
the sequence (amino terminus)-G-A-(carboxy terminus)
prepared as in Example 17 (wherein the N2 of guanine
carries an FMOC moiety, the N6 of adenine carries a
p-nitrophenethoxycarbonyl moiety, the backbone amino
terminus carries a t-BOC moiety, and the carboxy
terminus is in the form of a p-nitrophenyl ester) is
dissolved in a minimal volume of dry THF and added to

-93-

the glass support and mixing is carried out for 2 h (no catalyst is added for this or subsequent coupling steps). The support is washed with THF.

Any unreacted amine moieties are capped by
5   adding 2 ml of 2 M p-nitrophenyl acetate in THF and mixing at rt for 20 min. Thereafter, the glass support is washed with THF.

The terminal t-BOC moiety is removed with TFA and the support neutralized, as described above.

10  Subsequent dimeric subunits, prepared as in Example 17, are added in a like manner in the following order: (C-terminus) T-A, A-C, A-T, T-T, T-T, C-A, C-A, A-C (N-terminus).

Subunits having exocyclic ring nitrogen
15  protective groups removable by good nucleophiles (e.g., benzoyl for C, benzoyl or nitrobenzoyl for A, and acetyl or isobutyryl for G) can also be used for assembling polymers by the above procedures. However, polymers having these alternative protective groups are generally
20  deprotected by treatment with ammonium hydroxide rather than by DBU.


## Example 23

Configuring the carbonate-linked polymer of Example 20
25  for a solution application.

The 5' oxygen of the support-bound polymer of Example 20 is succinylated with 5 ml of a THF solution 1 M in succinic anhydride and 0.1 M in DMAP for 10 min at rt, washing with THF, and filtering. Next, the
30  polymer is both released from the support and its recognition moieties are deprotected by treatment with 3 ml of dry DMSO containing 0.3 g of DBU at rt for 12 h. The polymer is freed from DBU by two cycles of precipitation with anhydrous either and resuspension in

-94-

anhydrous DMSO. Thereafter, the polymer is resuspended
in aqueous DMSO containing a trace of pH 7.5 buffer to
neutralize any residual DBU and purified by anion
exchange chromatography.

5
## Example 24
### Configuring the carbonate-linked polymer of Example 20 for a solid phase application.

6-Aminocaproic acid is reacted with
10  2-phenylisopropylphenyl carbonate by the method of
Sandberg and Ragmarsson (Int J Peptide Prot Res (1974)
6:111). The N-protected (10 mmol) product is then
activated by reaction with 20 mmol of DCC and 50 mmol of
p-nitrophenol in a minimal volume of DMF. the resultant
15  product is purified on silica gel developed with ether.

The support-bound polymer of Example 20 is
reacted with 1 mmol of the above aminocaproic product
plus 0.1 mmol of DMAP in 2 ml of THF for 2 h at rt. The
glass support is then washed with THF and filtered. the
20  polymer is both released from the support and its
recognition moieties deprotected by treatment with 3 ml
of dry DMSO containing 0.3 g of DBU at rt for 12 h. The
polymer is freed from DBU by two cycles of precipitation
with anhydrous ether and resuspension in anhydrous DMSO.

25      The phenylisopropoxycarbonyl group is removed
by adding 80% aqueous acetic acid and incubating at rt
for 2 h. The deprotected polymer is precipitated with
ether, resuspended in aqueous DMSO, and purified by
cation exchange chromatography.

30      One gram of cellulose (Type 20, Cat. No. 3504,
average particle size of 20 microns, from Sigma Chemical
Co.), 1 g of bis(p-nitrophenyl) carbonate, and 0.2 ml of
triethylamine are added to 5 ml of DMF and stirred
overnight at rt. The slurry is filtered, washed once

-95-

with 10 ml of DMF, then with 50 ml of methanol, and
dried under vacuum.  A typical level of activation
obtained by this procedure is 23 µmol of
p-nitrophenoxycarbonyl moiety bound per gram of
5   cellulose.

The activated cellulose (0.5 g) is washed with
DMSO, filtered, and 10 µmol of polymer in 1 ml of DMSO
is added followed by 30 µmol of diisopropylethyl
amine.  This mixture is stirred slowly overnight at rt
10   in a closed vessel, filtered, and washed with DMSO.  The
support is next washed with aqueous pyridine, rinsed
with methanol, and dried.


Example 25

15   Configuring the carbamate-linked polymer of Example 21
for a solution phase application.

The 5' amino moiety of the support-bound
carbamate-linked polymer of Example 21 is succinylated
by reacting with 5 ml of a THF 1 M solution in succinic
20   anhydride and 1 M in diisopropylethylamine for 10 min at
rt, then washing with THF.

The polymer is released from the support by
treatment with 3 ml of DMF containing 0.3 g of DBU.  The
polymer is freed from the DBU by 2 cycles of
25   precipitation with ether and resuspension in DMF.

Protective groups are removed from the
recognition moieties by precipitating the polymers with
ether, resuspending in 1 ml of DMSO, and adding 1 ml of
concentrated ammonium hydroxide.  Generally, when the
30   protective group on adenine is a benzoyl moiety,
deprotection is carried out in a closed container at
30°C for 12 h.  Alternatively, when the protective group
on adenine is a nitrobenzoyl and the protective group on
guanine is an acetyl moiety, deprotection is carried out

-96-

at rt for 6 h.   Volatile components are removed under
vacuum and the polymer solution neutralized with pH 7.5
buffer.   Finally, the polymer, diluted in aqueous DMSO,
is purified by anion exchange chromatography.

5

### Example 26

Configuring the carbamate-linked polymer of Example 20
for a solid phase application.

The activated 6-aminocaproic acid reagent (1
10   mmol) of Example 23 in 3 ml of THF is reacted with the
support-bound carbamate-linked polymer of Example 21 for
20 min at rt.   The support is washed with THF and
filtered.   The terminal phenylisopropoxycarbonyl moiety
is removed by washing with dichloromethane, then
15   reacting with 5 ml of 0.5% trifluoroacetic acid in
dichloromethane for 15 min at rt, washing with THF, and
filtering.

The polymer is released from the support and
protective groups on the recognition moieties are
20   removed as in Example 25, except that the final
purification is by chromatography on a cation exchange
column.

The purified polymer is linked to a cellulose
support as in Example 20.

25

### Example 27

Configuring the amide-linked polymer of Example 22 for a
solution phase application.

The support-bound amide-linked polymer of
30   Example 22 is succinylated as in Example 24.

The polymer is released from the support, its
protective groups on the recognition moieties are
removed, and it is purified as in Example 24.

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## Example 28

Configuring the amide-linked polymer of Example 22 for a
solid phase application.

The support-bound amide-linked polymer of
Example 22 is reacted with activated aminocaproic acid
and the phenylisopropoxycarbonyl moiety removed as in
Example 24.

The polymer is released from the support, its
protective groups on the recognition moieties are
removed, it is purified, and then linked to a cellulose
support essentially as in Example 24.

While preferred embodiments of the invention
have been described herein, it will be appraent to those
in the art that various modifications and changes can be
made without departing from the invention.

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IT IS CLAIMED:

1.   A polymeric composition effective to bind, with a selected binding affinity, to a single-stranded polynucleotide containing a target sequence of bases, comprising non-homopolymeric, substantially stereoregular polymer molecules of the form:

$$R_1 \quad R_2 \quad R_3 \quad R_n$$

$$B \sim \cdot B \quad\quad B \sim \ldots B,$$
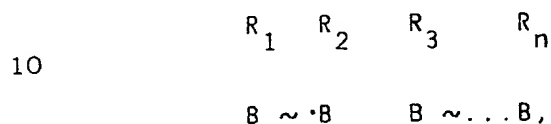
where:

(a) where $R_1$-$R_n$ are recognition moieties selected from purine, purine-like, pyrimidine, and pyrimidine like heterocycles effective to bind by Watson/Crick pairing to corresponding, in-sequence bases in the target sequence;

(b) n is such that the total number of Watson/Crick hydrogen bonds formed between a polymer molecule and target sequence is at least about 15;

(c) B    B are backbone moieties joined predominantly by chemically stable, substantially uncharged, predominantly achiral linkages;

(d) the backbone moiety length ranges from 5 to 7 atoms if the backbone moieties have a cyclic structure, and ranges from 4 to 6 atoms if the backbone moieties have an acyclic structure; and

(e) the backbone moieties support the recognition moieties at positions which allow Watson/Crick base pairing between the recognition moieties and the corresponding, in-sequence bases of the target sequences.

2. The composition of claim 1, wherein the
recognition moieties are selected from the following
group:
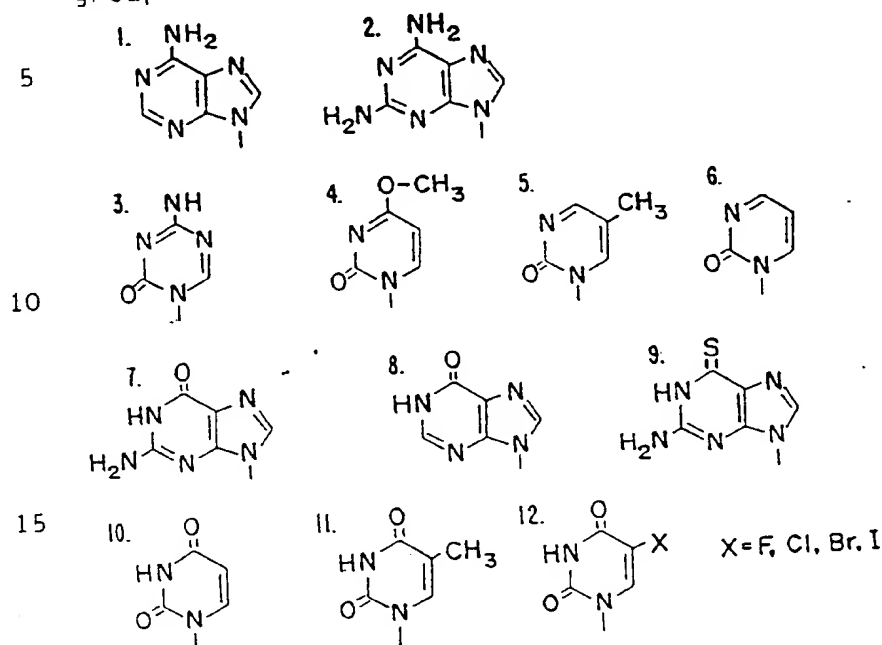


3. The composition of claim 1, wherein the
recognition moieties are selected from the following
group:
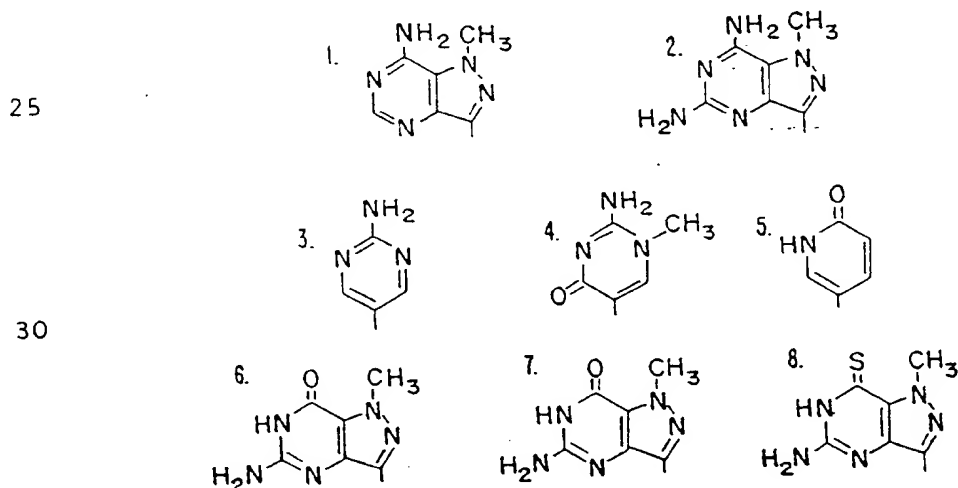
-100-

4.  The composition of claim 1, wherein B is a cyclic backbone moiety, and B ~ B has one of the following forms:

5



A-A.          B-B.          C-C.

10

15



D-D.          F-F.          G-G.

20

where Y = O, S and E = $\overset{O}{\underset{}{\overset{\parallel}{C}}}$ or $\overset{O}{\underset{\overset{\parallel}{\underset{O}{S}}}{}}$

25
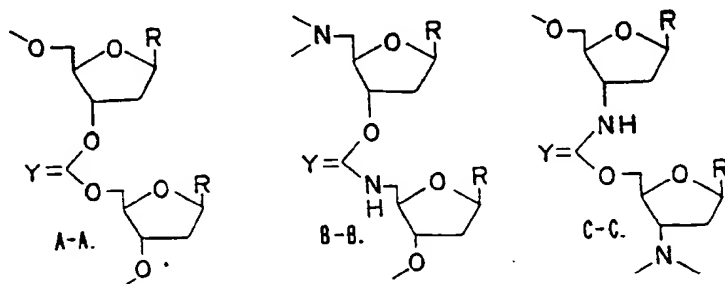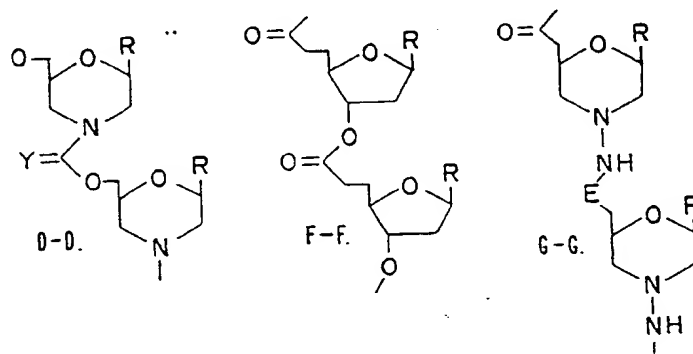
30

-101-

5.    The composition of claim 4, wherein B ~ B
has one of the following forms:

5



A-A.          B-B.          C-C.          D-D.

10
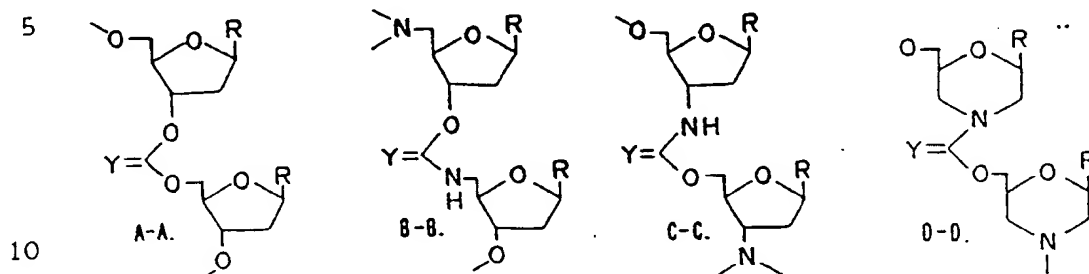
15

where Y = S, O.

6.    The composition of claim 1, wherein B is an
20  ayclic backbone moiety, and B ~ B has one of the
following forms:



I-I, L-L

25

J-J, M-M

30

K-K, N-N
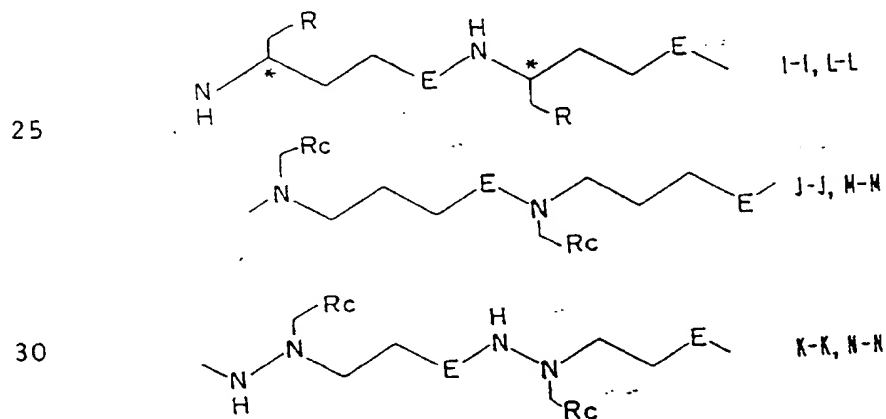
-102-

7. The composition of claim 1, for use in binding to an intracellular target sequence, wherein the polymer molecules contain at most 1-3 ionic charges, and no more that about 1 charge per 4 subunits.

5

8. The composition of claim 7, wherein the polymer molecules contain charged groups at one or both molecule ends.

10      9. A method of preparing a substantially uncharged polymeric composition effective to bind specifically and with a substantially uniform binding affinity to a single-stranded polynucleotide, said method comprising

15      (1) selecting a specific sequence of bases in the polynucleotide, said sequence constituting the target sequence;

      (2) Providing a group of subunits of the form: $B-R_i$, and $B-R_j$, where:

20      (a) $R_i$ and $R_j$ are recognition moieties selected from purine, purine-like, pyrimidine, and pyrimidine-like heterocycles effective to bind by Watson/Crick pairing involving two and three hydrogen bonds, respectively, to corresponding bases contained in

25 the selected target-specific sequence;

      (b) B is a backbone moiety which can be coupled to another backbone moiety B by a chemically stable, uncharged, achiral linkage;

      (c) the spacing between adjacent backbone

30 moieties, when coupled, is 6 atoms if the backbone moieties have a cyclic structure, and between 4-6 atoms if the backbone moieties have an acyclic structure; and

      (d) the backbone moieties, when coupled, form a backbone which supports the recognition moieties at

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positions which allow Watson/Crick base pairing between
the recognition moieties and the corresponding,
in-sequence bases of the target sequences; and

     (3) coupling the subunits in a defined sequence
5   corresponding to the sequence of bases in the
target-specific sequence.

     10.   The method of claim 9, which further
includes providing both $R_i$ and $R_j$ recognition
10  moieties for at least one base in the target-spepcific
sequence, and adjusting the binding affinity of the
composition for the target sequence by decreasing the
ratio of $R_i/R_j$, to increase the binding affinity,
and increasing the ratio, to decrease the binding
15  affinity.

     11.   The method of claim 9, which further
includes introducing a group which carried an ionic
charge at or near neutrality at one or both ends of the
20  polymer molecules, to increase the solubility of the
polymer in an aqueous medium.

     12.   The method of claim 9, wherein $R_i$ are
selected from the following group:

25



30
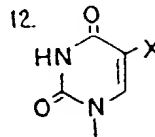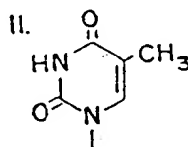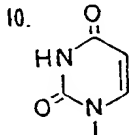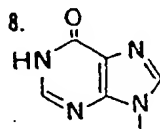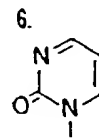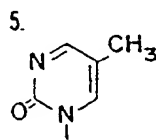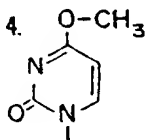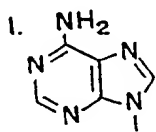
-104-

and $R_j$ are selected from the following group:
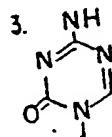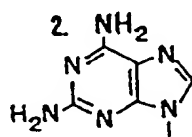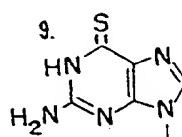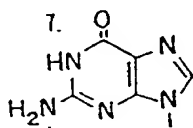


where X=F, CL, Br,  or I.

13. The method of claim 9, wherein B is a
cyclic backbone moiety, and adjacent backbone moieties,
when linked together, have one of the following forms:



where Y = S, O and E = $\overset{O}{\underset{}{\overset{\parallel}{C}}}$ or $\overset{O}{\underset{\downarrow O}{\overset{\parallel}{S}}}$
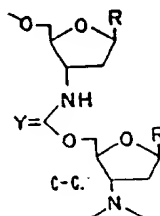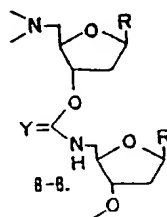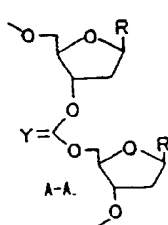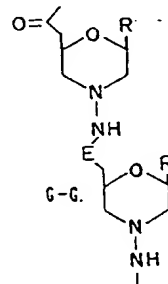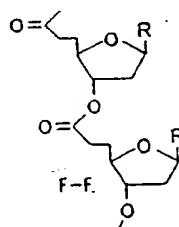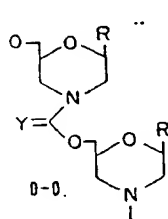
-105-

14.   The method of claim 9, wherein B is an
acyclic backbone moiety and adjacent backbone moieties,
when linked together, have one of the following forms:

5



10

15

$$\text{where } E = \overset{O}{\underset{}{\overset{\|}{C}}} \text{ or } \overset{O}{\underset{\underset{O}{\downarrow}}{\overset{\|}{S}}}$$

20
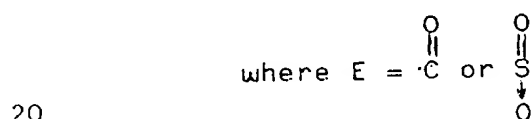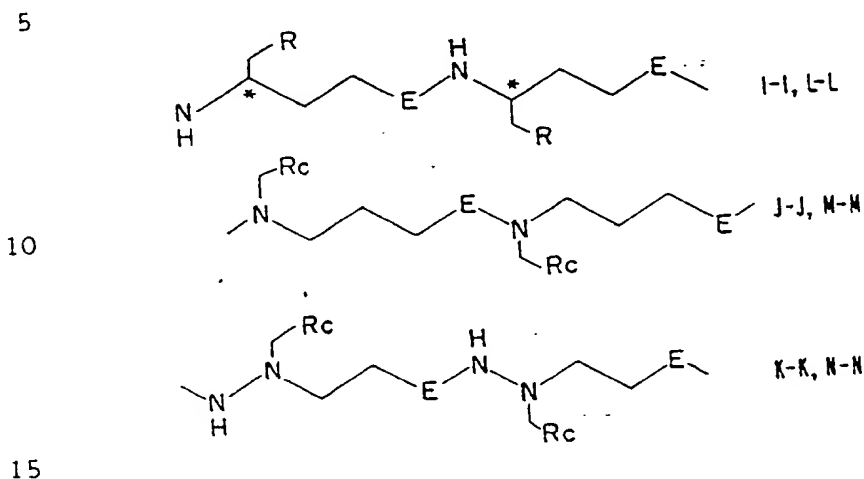
15.   A method for inhibiting expression of a
single-stranded polynucleotide contained in a system in
which a selected activity is expressed, said method
25  comprising,
         (1)selecting a target sequence in the
polynucleotide which is required for such expression,
         (2) providing a group of subunits of the form:
$B-R_i$, where:
30        (a) $R_i$ are recognition moieties selected from
purine, purine-like, pyrimidine, and pyrimidine like
heterocycles effective to bind by Watson/Crick pairing
involving two or three hydrogen bonds, to corresponding
bases contained in the selected target sequence;

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(b) B is a backbone moiety which can be coupled
to another backbone moiety B by a chemically stable,
uncharged, achiral linkage;

(c) the unit length of backbone moieties, when
5    coupled, is 5 to 7 atoms if the backbone moieties have a
cyclic structure, and 4 to 6 atoms if the backbone
moieties have an acyclic structure; and

(d) the backbone moieties, when coupled, form a
backbone which supports the recognition moieties at
10   positions which allow Watson/Crick base pairing between
the recognition moieties and the corresponding,
in-sequence bases of the target sequences;

(3)  coupling the subunits in a defined
sequence to form a predominantly uncharged, stereoisomer
15   polymer designed to bind specifically to the target
sequence,

(4) adding the polymer to the system and
observing the extent of inhibition of the polynucleotide
expression,
20         depending on the degree of inhibition of
polynucleotide expression which is observed, adjusting
the binding affinity of the polymer for the target
sequence by one or both of the  steps of (a) varying the
length of the target sequence and the corresponding
25   polymer length (b)  varying the ratio of recognition
moeities which form three and two hydrogen bonds with
corresponding in-sequence polynucleotide bases; and

by said adjusting producing a polymer whose
binding affintity is substantially the lowest affinity
30   which is required to effect such inhibition of
polynucleotide expression.

FIG. 1



FIG. 2

FIG. 3
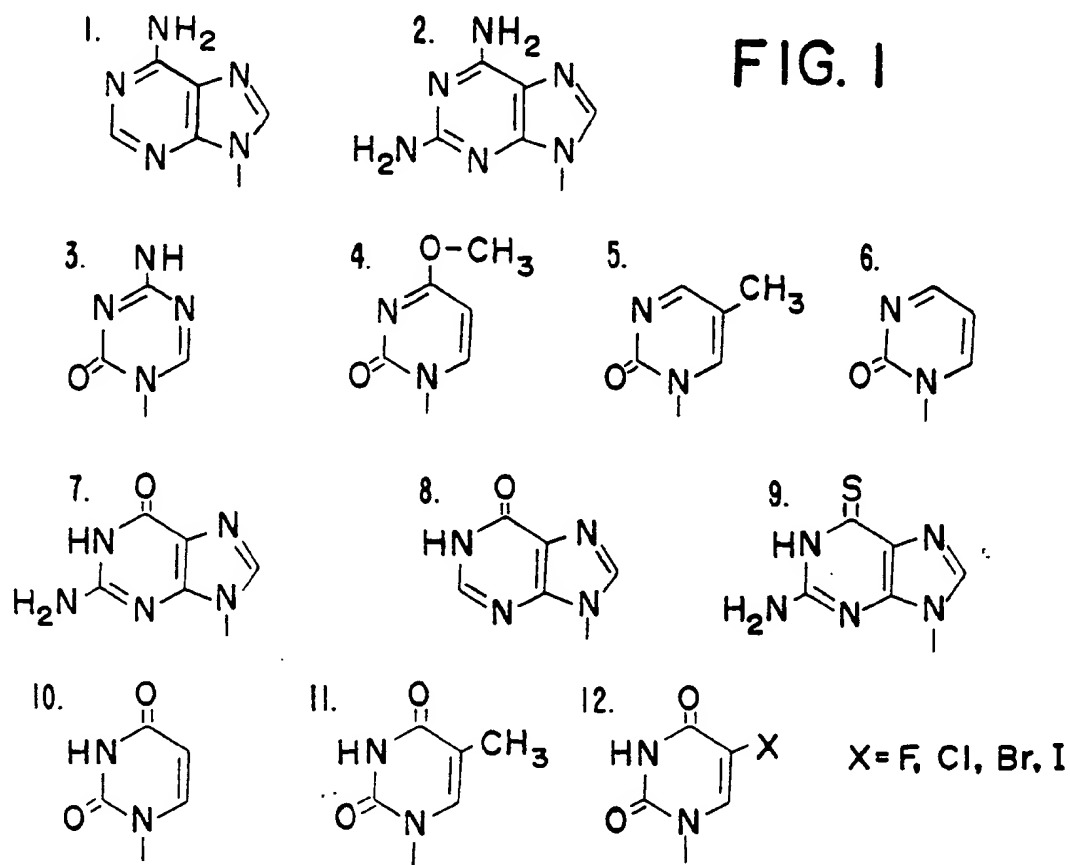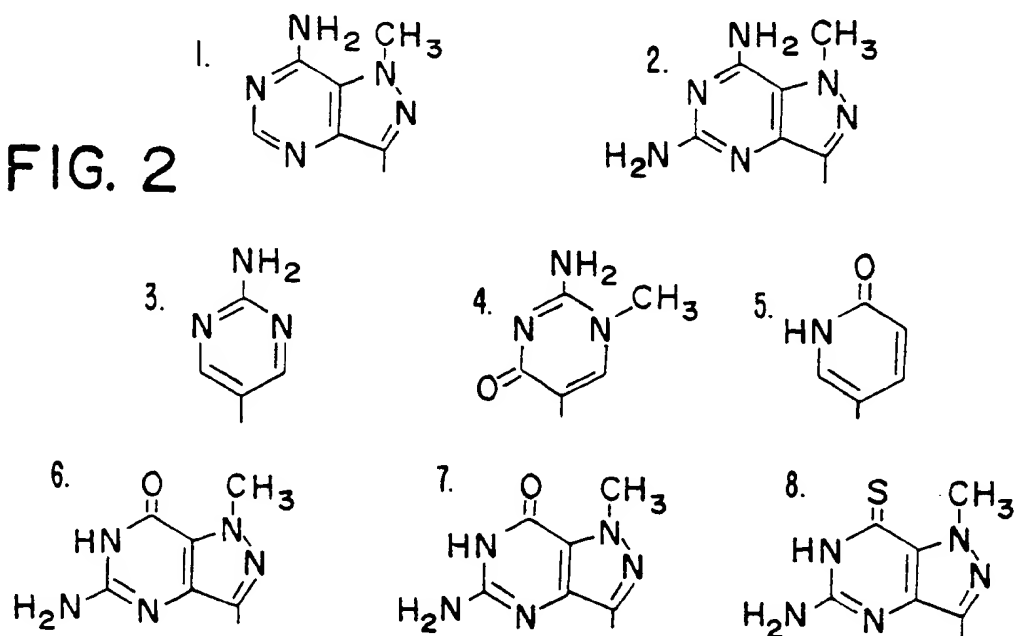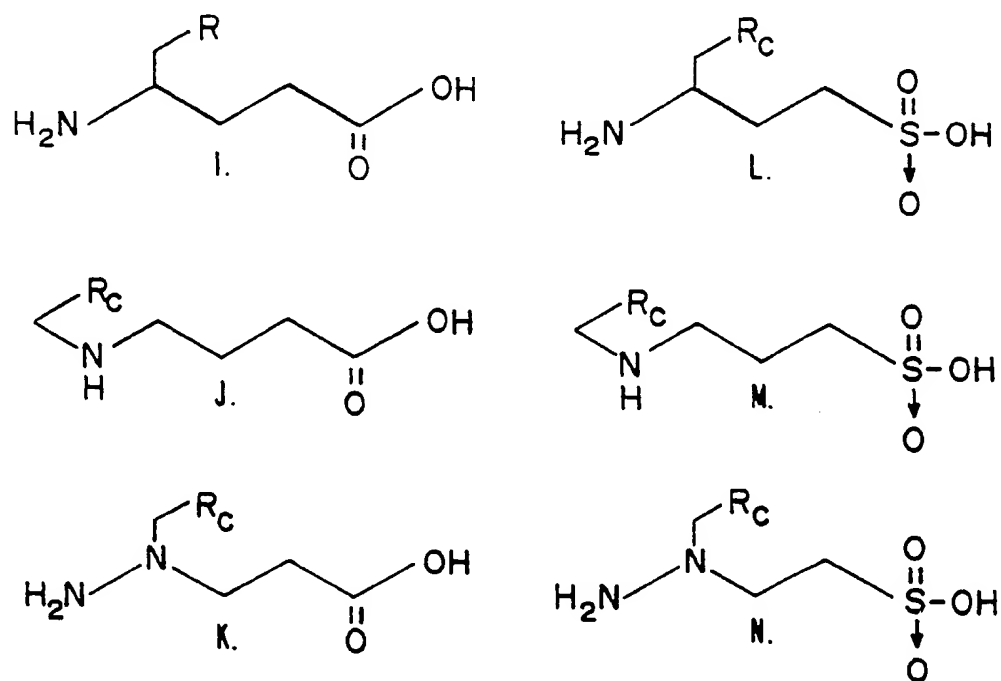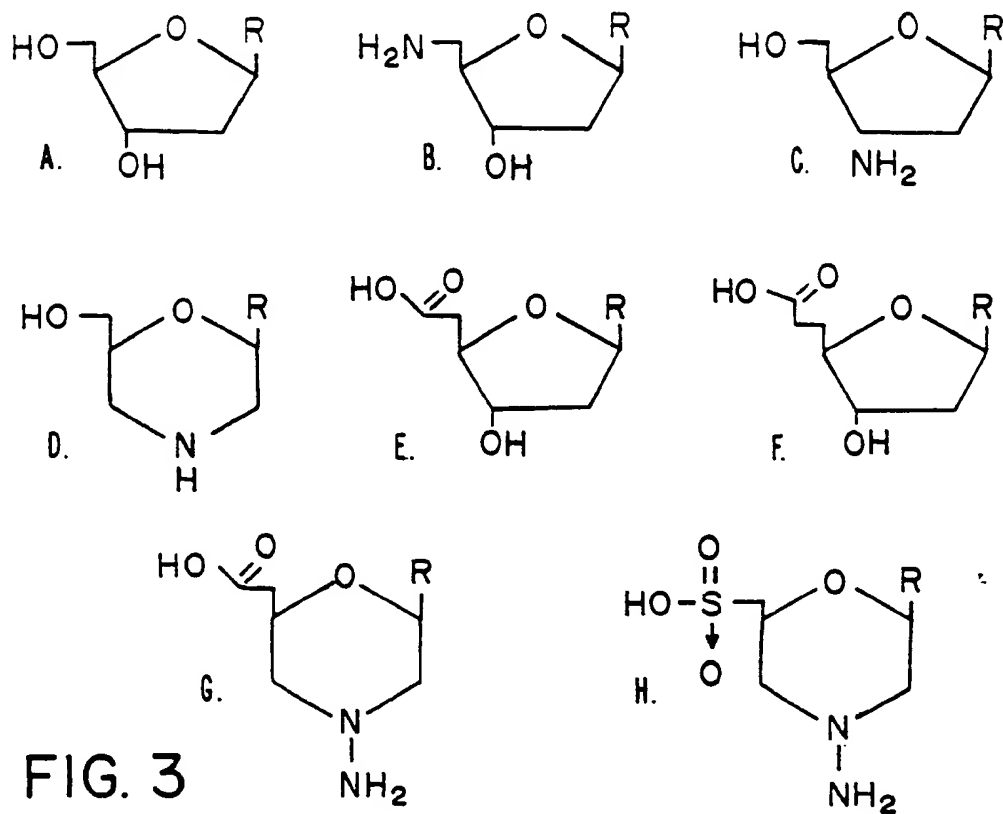


FIG. 4
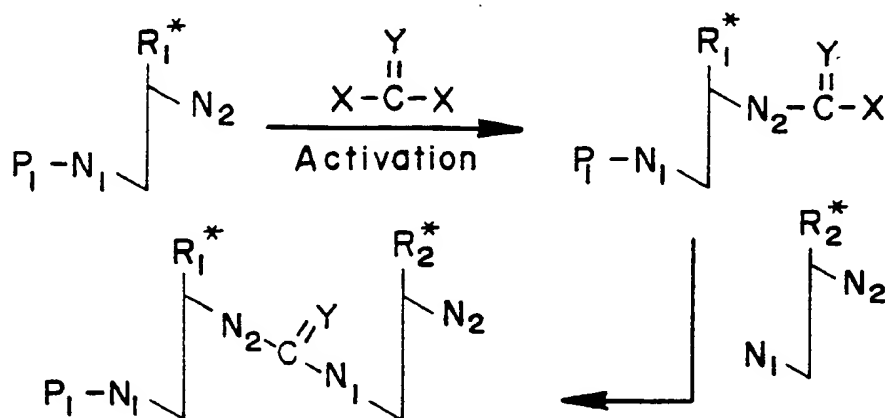
FIG. 5A
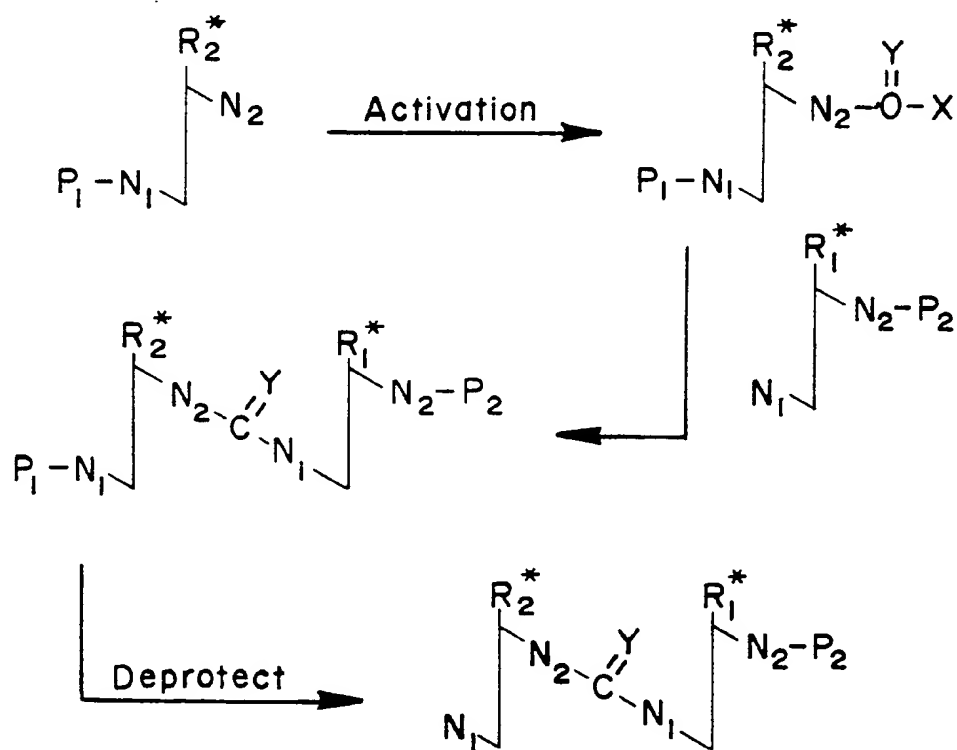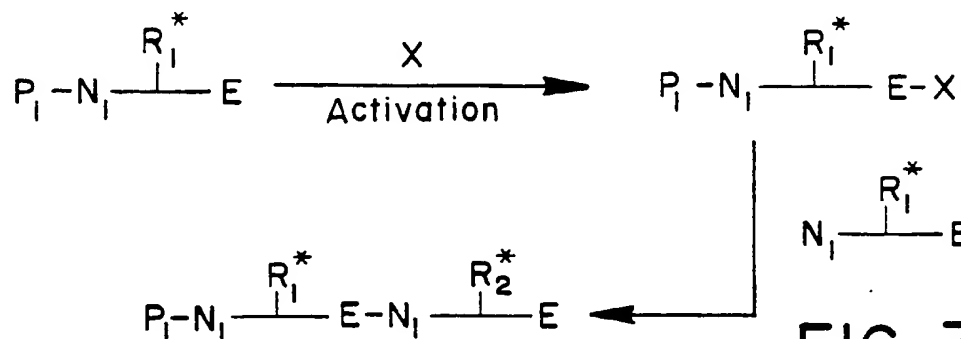


FIG. 5B
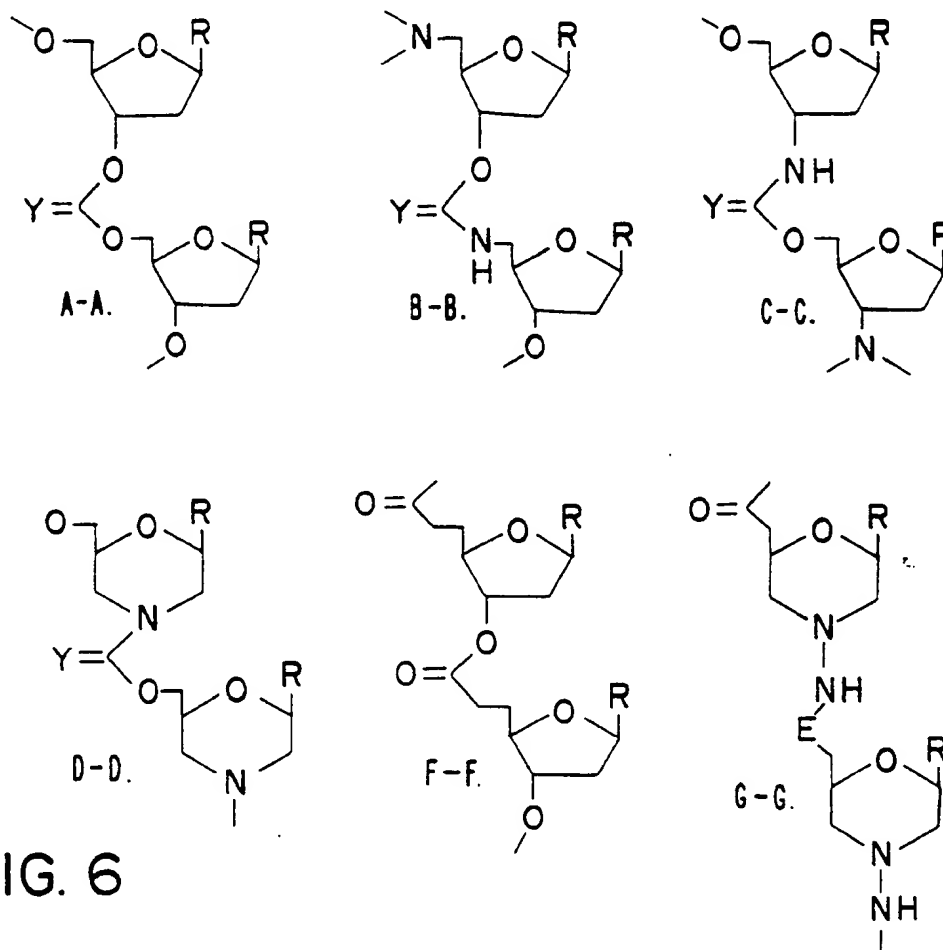
FIG. 6
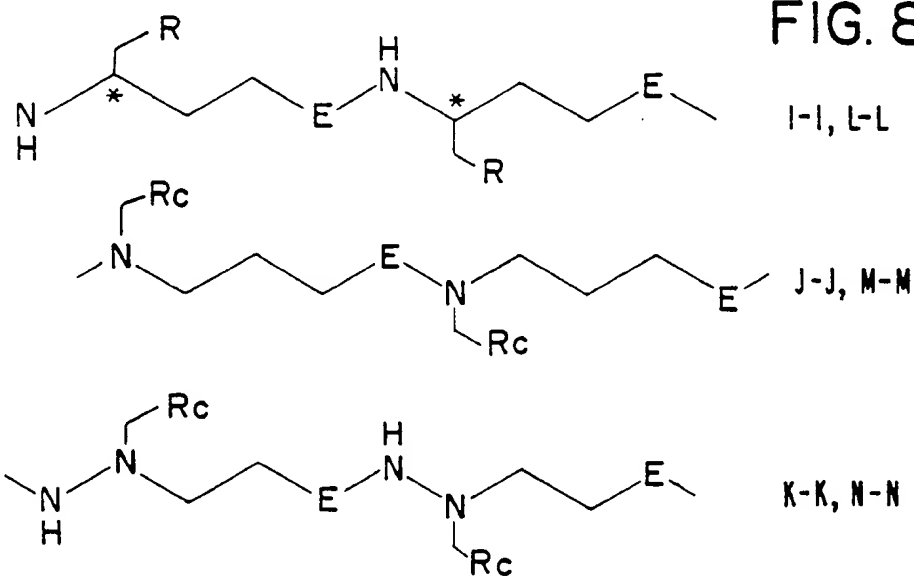
FIG. 8

FIG. 7A
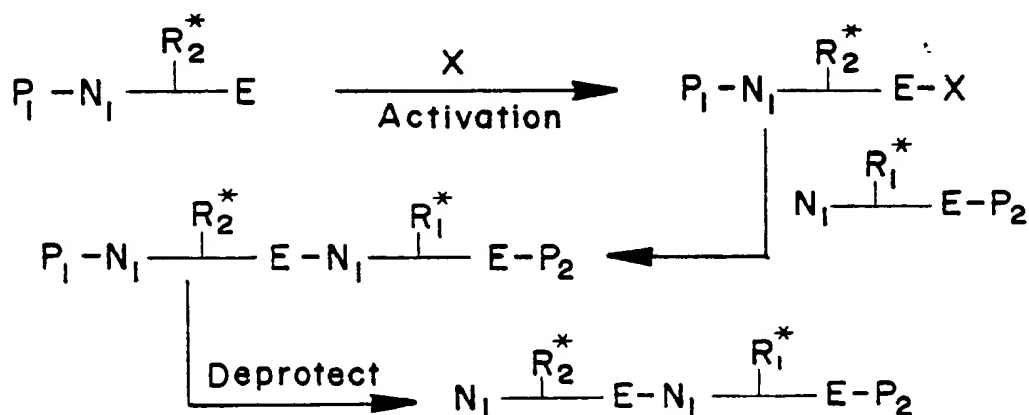


FIG. 7B
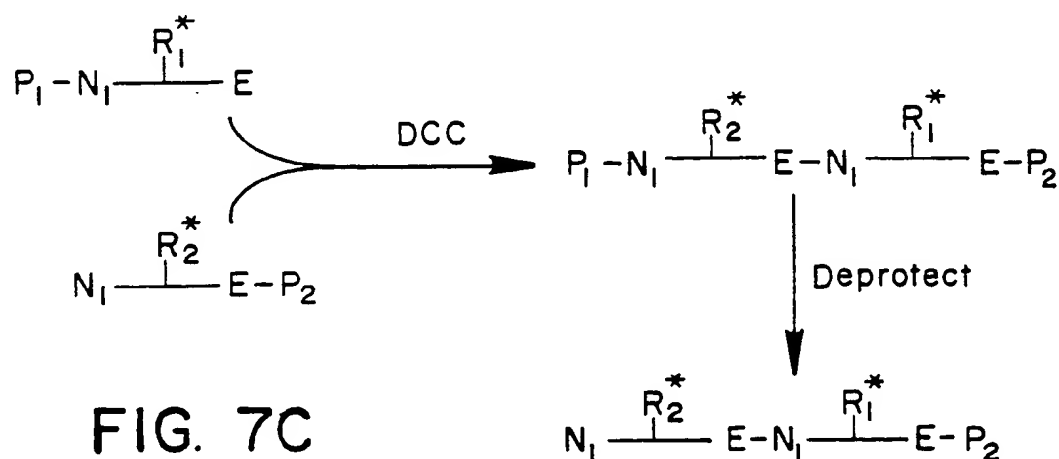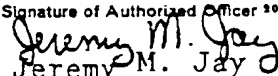


FIG. 7C

# INTERNATIONAL SEARCH REPORT

International Application No PCT/US86/00544

## I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

INT. CL.[4] : C12Q 1/68; C12P 19/34; C12N 15/00
U.S. CL. : 435/6,91; 935/44,78

## II. FIELDS SEARCHED

Minimum Documentation Searched [4]

| Classification System | Classification Symbols |
|---|---|
| U.S. | 435/6,68,91,188,810<br>436/94,501,518,808<br>514/44;  935/3,6,44,78 |

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched [5]

Computer Search: Chemical Abstracts 1967-1986;
Biosis 1977-1986; Derwent (WPI,WPIL,Biotechnology)

## III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

| Category [*] | Citation of Document, [16] with indication, where appropriate, of the relevant passages [17] | Relevant to Claim No. [18] |
|---|---|---|
| Y | Biochemistry, Volume 18, No. 23, issued November 13, 1979, P.S. Miller et al, "Nonionic Nucleic Acid Analogues. Synthesis and Characterization of Dideoxyribonucleoside Methylphosphonates," see page 5140 | 1-15 |
| P,Y | Biochemistry, Volume 24, No. 22 issued August, 1985, A. Murakami et al, "Characterization of Sequence-Specific Oligodeoxyribonucleoside Methylphosphonates and Their Interaction with Rabbit Globin mRNA," see pages 4045 and 4046. | 1-15 |

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

## IV. CERTIFICATION

| Date of the Actual Completion of the International Search [3] | Date of Mailing of this International Search Report [3] |
|---|---|
| 03 JUNE 1986 | 2 3 JUN 1986 |

| International Searching Authority [1] | Signature of Authorized Officer [20] |
|---|---|
| ISA/US | Jeremy M. Jay |

Form PCT/ISA/210 (second sheet) (October 1981)

| III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET) | | |
|---|---|---|
| Category * | Citation of Document, [16] with indication, where appropriate, of the relevant passages [17] | Relevant to Claim No [18] |
| Y | Chemical Abstracts, Volume 91, No. 19, issued 1979 (Columbus, Oregon, USA), J. Summerton, "Intracellular inactivation of specific nucleotide sequences: a general approach to the treatment of viral diseases and virally mediated cancers," see pages 73, column 2 and 74, column 1, the abstract No. 151527g, J. Theor. Biol. 1979, 78(1), 77-99 (Eng). | 15 |
| Y | US,A, 4,123,610 (Summerton) 31 October, 1978, see column 3, lines 3-11. | 15 |
| Y | Nucleic Acids Research, Volume 12, No. 8, issued April 25, 1984, M. Renz et al, "A colorimetric method for DNA hybridization", see page 3435. | 1-14 |
| Y | Biochemistry, Volume 20, No. 7, issued March 31, 1981, P.S. Miller et al, "Biochemical and Biological Effects of Nonionic Nucleic Acid Methylphosphonates", see pages 1878 and 1879. | 1-15 |
| Y | US,A, 4,507,433 (Miller) 26 March 1985 see column 1, lines 32-37. | 1-15 |
| Y | Journal of Biological Chemistry, Volume 255, No. 20, issued October 25, 1980, P.S. Miller et al, "Oligothymidylate Analogues Having Stereoregular, Alternating Methylphos-phonate/Phosphodiester Backbones", see page 9659. | 1-15 |